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Interferon Induced Transfer of Viral Resistance

Annual and Final Report

J. Edwin Blalock, Ph.D.

February 1982

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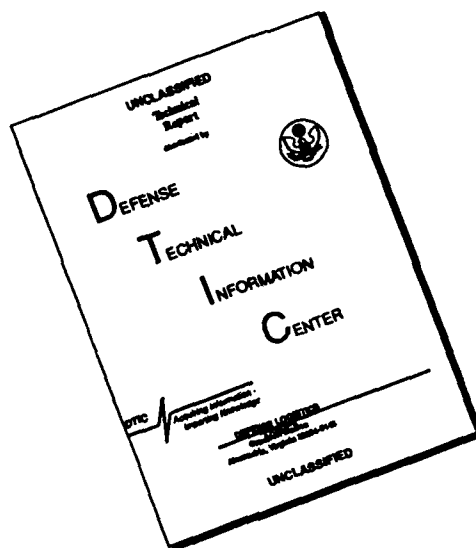
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During this granting period, we have found that: (a) transfer of viral resistance within a cell line occurs for about 4 hrs after IFN addition; (b) in the transfer of viral resistance by lymphocytes, with the exception of erythrocytes, virtually any allogeneic or xenogeneic tumor cell or normal xenogeneic cell induces leukocyte interferon (IFN- α) production by B but not T lymphocytes; (c) once IFN- α is produced, it initiates transfer by both B and T lymphocytes; (d) lymphocyte transfer is probably mechanistically the same as transfer by fibroblastoid and epithelioid cells; (e) the "foreign cell" inducer for IFN- α is a cell surface glycoprotein; (f) the lymphocyte receptor for the IFN- α inducer is a cell surface protein; (g) null cells are the probable source of the IFN induced immunosuppressive factor; (h) IFN induces melanogenesis and steroidogenesis; (i) adrenocorticotrophic hormone (ACTH) has antiviral activity on its target cells; (j) ACTH and endorphin are produced by lymphocytes in the form of IFN- α ; (k) IFN- α production by lymphocytes can be studied with antisera to ACTH or endorphins; (l) fibronectin has antiviral activity which is mediated by induction of a new protein; (m) IFN induces fibronectin; (n) fibronectin may be related to the IFN induced transfer material.

These findings show that: (a) transfer of IFN induced viral resistance by lymphocytes involves a new system of recognition of foreignness; (b) both B and T lymphocytes disseminate IFN's antiviral activity; (c) null cells disseminate the immunoregulatory activity of IFN; (d) interferon has broad hormonal activity; (e) hormones may function as antiviral substances; (f) there may be a regulatory circuit between the immune and neuroendocrine system with IFN- α as an intermediary (g) fibronectin may be involved in the antiviral action of IFN.

The understanding of the molecules involved in the aforementioned processes may lead to a new series of antiviral and immunosuppressive substances as well as a possible new strategy of tissue targeted antiviral and antitumor therapy.



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ABSTRACT

The overall objective was the continued study of the cells, mechanisms and molecules involved in the transfer of interferon (IFN) induced viral resistance, immunosuppression and hormonal activity. Studies during the first two granting periods led to a probable model that an IFN-donor cell membrane interaction generates a secondary messenger molecule(s) which transmit the IFN signal from the membrane to the nucleus. This molecule(s) in turn is transferred to adjacent recipient cells and causes derepression of their genes for the antiviral protein.

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The understanding of the molecules involved in the aforementioned processes may lead to a new series of antiviral and immunosuppressive substances as well as a possible new strategy of tissue targeted antiviral and antitumor therapy.

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RESEARCH PLAN

A. INTRODUCTION

I. Objective:

The overall objective of this research proposal was the continued study of the cells, mechanisms and molecules involved in the transfer of interferon induced viral resistance, immunosuppression and hormonal activity.

1. What are the kinetics of transfer of viral resistance within a population of cells?
2. What cell types induce leukocyte interferon (IFN_α) production by non-sensitized lymphocytes?
3. What are the characteristics of the IFN_α inducing factor from foreign cells?
4. Is there a receptor on non-sensitized lymphocytes which recognizes the IFN_α inducing factor?
5. What are the characteristics of the receptor for IFN_α induction?
6. Which lymphoid cell types transfer IFN induced viral resistance?
7. What cellular and molecular events are involved in lymphocyte transfer of IFN induced viral resistance?
8. Which lymphoid cell type(s) produces the IFN induced immunosuppressive factor?
9. What is the functional relationship of IFN and polypeptide hormones?
10. What is the structural relationship of IFN and polypeptide hormones?
11. Does fibronectin have antiviral activity?
12. What are the characteristics of the antiviral activity of fibronectin?

II. Background:

Interferon shares a number of similarities with polypeptide hormones (1). For instance, penetration of the cell membrane is not required for its action (2,3). The interferon-membrane interaction in turn leads to derepression and production of the antiviral protein (4,5). Prior to the work herein reported, essentially nothing was known about the events between interferon action at the cell membrane and derepression of the gene for the antiviral protein. We designed a system for the study of these events (6). This system was based on two observations. First, that many animal cell types exhibit the ability to communicate between themselves in vivo and in vitro (7). This communication is thought to occur through gap junctions which allow cells to share their metabolites and small control molecules (8-12). Second, the action of polypeptide hormones on transcriptional and translational processes are mediated by secondary molecules which are produced in response to a hormone-cell membrane interaction (13). We hypothesized that if, as the case with polypeptide hormones, the induction of the antiviral protein is mediated via secondary molecules, these might influence adjacent cells. The many instances of the species specificity of interferon action (14) made this a testable

hypothesis. Briefly, we found that cells made resistant to virus infection by treatment with their homologous interferon can transfer viral resistance to cells of a heterologous species insensitive to that interferon (6). For instance, while human WISH or baby hamster kidney cells (BHK) alone were not sensitive to the action of mouse interferon, cocultivation of these cells in the presence of mouse interferon and sensitive mouse L cells resulted in a marked inhibition of the expected yield of virus from the interferon insensitive cells. Control cell mixtures, in the absence of interferon, yield at least the expected amount of virus as compared with the yield from either cell type alone. The controls showed that inhibition of virus yield does not result from cocultivation of different cell species but resulted from the presence of the mouse interferon preparation with the cocultivated cells. The transfer of resistance was dependent on cell proximity since the degree of transfer was controlled by both the donor (L) to receptor (WISH or BHK) cell ratio as well as the absolute cell density at a given ratio. Interferon-induced transfer of viral resistance was not observed until the majority of cells were in close contact with neighboring cells. Using poliovirus (which infects only human cells) (15), we have directly shown that the human WISH cell cocultured with mouse L cells in the presence of mouse interferon is protected and that this protection is dependent on the interferon dose. These data seem to confirm the hypothesis that the presence of interferon with its homologous cell can induce antiviral activity in heterologous cells and suggest that cell-to-cell communication can be demonstrated with interferon action.

We have examined the possible mechanism(s) governing the transfer of interferon-induced viral resistance between heterologous cells (16). The possible mechanisms include: (a) interferon production by the recipient cells; (b) transfer to the recipient cells of sensitivity to heterologous interferon possibly through transfer of a membrane receptor; (c) transfer of a putative secondary messenger molecule(s) which transmits a derepression signal between the cell membrane and the nucleus; (d) transfer of the mRNA for the antiviral protein; and (e) transfer of the antiviral protein. The available evidence indicates that transfer of viral resistance from interferon-treated mouse L cells to human WISH cells does not result from the production of human interferon by human WISH cells. This idea is supported by the findings that VERO cells, which produce essentially no interferon (17) receive transferred resistance from L cells (18). Additional evidence arguing against human interferon production is the finding that transfer of virus resistance occurred to the same extent in the presence of antisera to human fibroblast interferon. Also consistent with this finding is the fact that transfer occurs during conditions of a single cycle of VSV growth which allows little time for interferon production and action. Although, there was a diminution in the amount of transferred resistance with increasing input m.o.i. of VSV, this was also seen with L cells alone and indicates that the resistance, once transferred, has the characteristics of an interferon-type antiviral state. Taken together these data seem to negate the production of interferon by the recipient cells as the basis for transfer of resistance.

Human WISH cells might be made sensitive to mouse interferon when cocultivated with mouse L cells by transfer of membrane receptors for interferon. However, this seems unlikely since this mechanism would require the presence of mouse interferon with the recipient WISH cells and we found that after a brief interaction of L cells with mouse interferon, followed by removal of the interferon, resistance was transferred to subsequently added WISH cells. Thus resistance transfer did not require the presence of mouse interferon with the human WISH cells.

Data was presented which showed that the development of resistance in the donor L cells precedes the development of resistance in the WISH cells (16). This suggests that the mouse interferon initiates an antiviral process in the L cells which is subsequently transferred to the human WISH cells. Theoretically, any one of the following molecules [putative secondary messenger(s), the mRNA for the antiviral protein or the antiviral protein] could be the effector molecule for transferred resistance.

If the transfer process occurs through gap junctions which transfer only small molecules (19), then it seems unlikely that mRNA or the antiviral protein is responsible. Data indicate that the interferon-induced material which is responsible for the transfer of resistance is unstable or it becomes unavailable for transfer (16). Since the mouse antiviral protein is stable for more than 8 h (20) and its production continues in the presence of interferon, it seems unlikely that it alone is responsible for resistance in the WISH cells. Thus either a molecule other than the antiviral protein, or the antiviral protein plus another factor (which is no longer available by 8 h) is responsible for the transfer.

The mRNA for the antiviral protein also seemed an unlikely candidate for the effector of transferred resistance. If the mouse mRNA alone caused the viral resistance in the WISH cells, then actinomycin D should not have blocked development of resistance in the WISH cells beyond the 1 h required for substantial transcription of the mRNA in the L cells (16). Since actinomycin D blocked resistance in the WISH cells for 3 h past its effect on L cells, these data imply that a transcription event in the WISH cells is necessary for the development of the antiviral state. These findings also argue against the transfer of the antiviral protein, since its possible action in WISH cells should not require transcription. Again, the more complex possibility that actinomycin D blocks the production of a factor needed to transfer the mRNA cannot be excluded. By a process of elimination and in light of the data with actinomycin D, secondary messenger molecules which transmit the interferon signal from the membrane to the nucleus are favored as the effector substance(s) for the transfer process leading to derepression of the gene for the human antiviral protein. This model for the transfer phenomenon is strengthened by our preliminary findings of a soluble interferon induced material from L cells which confers viral resistance on human WISH cells.

To be certain that the transfer phenomenon was not limited to a few cell types, we examined other cell species and their ability to exhibit transfer. We have shown that this phenomenon also occurs when rabbit kidney and human WISH cells, with their corresponding interferons, are cocultivated

with human WISH and baby hamster kidney cells, respectively. This finding increases the number of donor cell types to three. The related finding that monkey VERO and chick embryo cells can be recipients of transferred resistance expands the number of heterologous recipient cell species to five (18). With a fairly large number of cell species demonstrating the transfer of resistance, this could possibly be a general phenomenon among adjacent cells within the body and thus play an important role in the pathogenesis of viral infections. Information relevant to this idea came from the finding that the rate of development of interferon-induced virus resistance in a mixture of two human cell types (U and WISH) is determined by the cell type (WISH) in the mixture which responds first (21). The transfer of virus resistance from one human cell (WISH) to another (U) (homologous transfer) is much more efficient than the transfer from mouse L cells to WISH cells (heterologous transfer), as was shown by a much lower ratio of donor to recipient cells required for maximum transfer as well as a more rapid transfer. Thus, virus protection afforded by the interferon system is amplified more efficiently in mixtures of different human cells than in mixtures of mouse and human cells (21). An explanation for this difference in efficiency might be found in the mechanism of transfer between cells. For example, if transfer of virus resistance occurs through gap junctions which allow cells to communicate between themselves, then the efficiency of the transfer should be a reflection of the relative ability of the cells to communicate. Recently, specificity of junctional communication was shown and appeared to occur more frequently between homologous than heterologous cells (11, 12). Hence, the demonstration that a lower percentage of donor cells is required in a homologous cell mixture than in a heterologous cell mixture for maximum transfer of virus resistance may be explained in terms of the relative ability of these cells to communicate.

We have proposed that the natural action of interferon does not require a direct effect of the molecule on each cell. This proposition stemmed from our previous demonstrations of the transfer of interferon-induced viral resistance between cells (6). Supportive of this was the finding that interferon action was determined by the cell density (22). At a cell density where the majority of cells in a population could not contact one another there was a precipitous drop in interferon activity. This cell-proximity effect was proposed to result from variation in interferon sensitivity between individual cells in the population and an inability of the most sensitive, first-responding cells to transfer their viral resistance to less sensitive, slower-responding cells when they were not in contact. By the cloning of individual L cells it was shown that there is a very marked heterogeneity among individual cells in both their sensitivity to and maximum degree of protection afforded by interferon. Further, cloned L cells of "high" interferon sensitivity can transfer their viral resistance to clones of "low" sensitivity. By studying the interferon response of individually reacting clones and a reconstituted parental population of cells, it was found that as few as 10% of these cells can determine the response of the population (23). These findings strongly support our interpretation of the cell proximity effect and our contention that the action of interferon does not require a direct effect of the molecule on each responding cell. The observation of transfer among cells within a population from a single species suggests that the transfer process is operational in vivo. This is all the more likely since the cell proximity effect occurs with primary mouse embryo cells as well as diploid human fibroblasts (which are similar to normal cells in vivo) (22). This means that

the natural mechanism of interferon protection probably includes action on cells near the interferon-responding cell. This process would amplify the interferon response since sensitive, fast-responding cells would transfer resistance to less sensitive, slower-responding cells which constitute the bulk of the population. The observation that a small fraction of cells (10%) determines the response of the population shows that the transfer process plays a highly important role in the action of interferon. It also implies that the transfer probably proceeds through more than one recipient cell since one cell can only be surrounded by 6 cells in this system. Shortly after our demonstration of cell-to-cell communication of interferon activity, a similar finding with polypeptide hormones was reported (24). This leads to the intriguing possibility that as with interferon there may be marked heterogeneity among "sensitive" cells in their response to polypeptide hormones and only a small number of cells determine the response of the population (or in vivo the tissue). If this is correct then direct cell-to-cell communication represents a novel mechanism for the amplification of hormone or hormone-like activity. Also, this transfer process and the finding of large variations in sensitivity of cloned cells to interferon has the appealing aspect that all cells do not have to expend their cell machinery to a maximum extent for maximum sensitivity to interferon. This process would tend to be conservative for such things as cellular receptors for interferon.

In a heterologous transfer system only a fraction (30%) of L cell clones can transfer resistance to human WISH cells. Whether the same clones are responsible for homologous and heterologous transfer of viral resistance is not known. The donor cell phenotype seems to be an unstable characteristic and is therefore probably under epigenetic control. It is of interest to understand what controls the ability of cells to communicate since this is such an important system for coordination of functions within tissues. Phenotypically, we have observed a correlation between L cell colonial morphology and the ability to transfer resistance. The processes underlying the dense colony phenotype and its involvement in transfer may be helpful in understanding the control of cell-to-cell communication. Interferon sensitivity, of course, is a prerequisite to ability to transfer viral resistance. However, interferon sensitivity does not appear to control the ability of cells to communicate.

In addition to virus type interferons (α and β -IFN), immune-type interferon (IFN- γ), a lymphokine, can cause the transfer of viral resistance from mouse to human cells (25). This is similar to findings for virus-type interferon (6), except that immune-type interferon caused the transfer more efficiently. The immune-type interferon molecule was found to be the most likely substance in the interferon preparation to be responsible for the transfer. The transferred resistance had the characteristics of an interferon-induced antiviral state. The kinetics of development of transferred viral resistance in response to mouse immune-type interferon suggest that an antiviral process is initiated in the mouse cells and is subsequently transferred to the human cells. Interestingly, although the kinetics of the response of L cells to virus-type and immune-type interferon are different, there is a similar delay in the development of transferred resistance in the human WISH cells. This indicates that the transfer process and its expression in WISH cells may be similar with both interferon types whereas, the initial events by which the

two interferons activate L cells may be different. These data also indicate that within L cells, immune-type and virus-type interferons probably share some common pathways to the antiviral state. These findings further suggest that, like virus-type, some component(s) of the immune-type interferon system is (are) not species specific because immune-type interferon-treated L cells can transfer viral resistance to human WISH cells. The finding that immune-type is better able than virus-type interferon at eliciting the transfer mechanism points to a new and efficient means of disseminating the interferon response of lymphocytes.

Lymphoid cells have also been found to transfer interferon induced resistance to other cell types (26). A prerequisite to this transfer is the production of leukocyte interferon (IFN- α) by lymphoid cells in response to co-culture with heterologous cells (27). The present findings are the first demonstration that foreign cells stimulate non-sensitized lymphocytes to produce a type of interferon which has the properties of IFN- α . Further this induction of lymphocytes does not necessarily require transformed cells since normal heterologous cells also induce. The inducing component of the foreign cell does not require ongoing RNA and protein synthesis since they induce after treatment with actinomycin D. Additionally, intimate and or brief contact between the lymphocytes and the foreign cells, but not mycoplasmas or endogenous viruses, appears to be required for induction. The kinetics of production of leukocyte interferon by nonsensitized lymphocytes in response to foreign cells is similar to that induced by viruses. We have shown that a component probably of the foreign cell membrane, can be solubilized and in this state cause the induction of interferon by non-sensitized human leukocytes. The nature of the interferon inducer is important for several reasons. First, it initiates interferon production and thereby sets into motion leukocyte transfer of viral resistance. Second, it may prove to be a valuable in vivo interferon inducer. Third it is possible that many of the in vivo interferon inducers (e.g., bacteria, protozoa, and intracellular parasites including viruses which alter cellular antigens) induce by this mechanism. This is readily testable by characterization of the interferon type stimulated by the various inducers. Finally, a new cellular system for the recognition of foreignness is suggested by the ability of nonsensitized lymphocytes to recognize a foreign cell component and respond with a particular type of interferon. This may be of value in the diagnosis of certain tumors and infections. Furthermore, it may be a potential system for the easy production of virus-free, high-titered, human, leukocyte-type interferon.

It appears that a B cell is responsible for interferon production in our system (28). This is a new finding which may be of broad interest in terms of recognition of foreignness and cell-to-cell interactions in the immune system. After IFN- α is produced we have shown that human leukocytes can transfer interferon induced viral resistance to xenogenic cells of fibroblast and epithelial origin (26). The possible induction of an antiviral state in the recipient cells by endogenous interferon seems unlikely because no interferon to recipient cells was detected in supernates of any of the systems used and it is unlikely that mycoplasma or viruses were present in the primary chick embryo or secondary mouse embryo cultures that were used in this system. The transfer was rapid, efficient (1:1 cell ratios), and occurred only in the presence of leukocyte interferon. Transfer did not occur when the recipient cell did not induce interferon in the leukocytes. The antiviral state in the

xenogenic recipient cell had a characteristic of the antiviral state directly induced by the interferon in syngeneic or allogeneic cells. Specifically it was broadly active against viruses. The reduction of virus yields observed in recipient cells was not due to natural killer cell activity based on the amount of specific ^{51}Cr release, trypan blue dye exclusion, and uptake of neutral red dye observed during the critical parts of the studies and the small ratio of leukocyte to recipient cells required to induce the antiviral activity.

Lymphoid cells were also shown to transfer interferon's immunosuppressive activity (29). The indirect immunosuppressive action of interferon was found to be through induction of suppressor cells which in turn produce a suppressor factor that inhibits antibody production. We have the following working model of the production and function of the suppressor factor. Its induction is blocked by treatment of interferon with specific antibody, but the immunosuppression by induced suppressor factor is unaffected by antibody to interferon. The factor is devoid of antiviral activity, which suggests that interferon regulates the immune response by a mechanism(s) that is different from its antiviral property. This differentiates the cell interactions that are involved in immunosuppression by interferon from the cell-to-cell interactions that are associated with the transfer of viral resistance (6). Additionally, efficient transfer of viral resistance requires cell-to-cell contact, which is not required in immunosuppression. This suppressor factor may play a natural role in both normal immune mechanisms and in the host response to viral infections. It may also be a desirable means of suppressing the immune response under certain conditions.

The induction of a suppressor factor by interferon, which lacks antiviral activity, is consistent with two previous observations which suggest dissociation of the antiviral and immunoregulatory actions of interferon. One is the observation that the immunosuppressive effects of fibroblast interferon are blocked by 2-mercaptoethanol, while the antiviral property is unaffected (30). The other is that a ribosome-associated factor(s) obtained from interferon-treated cells is immunosuppressive, but lacks antiviral properties (31). One of the biochemical effects of interferon on cells has recently been shown to be a block of protein synthesis via blockage of formation of initiation complex through ribosome-associated protein kinase activity (31, 32). To date the only biological function that this mechanism has been shown to possibly affect is suppression of the immune response (31). It is quite possible, then, that interferon-induced molecular events such as inhibition of initiation complex formation and suppressor factor induction may be related to the non-antiviral properties of interferon.

As indicated above, the action of interferon is similar to polypeptide hormones and is probably mediated through secondary messenger molecules which influence adjacent cells. Subsequent to our work, the cell-to-cell transmission of hormonal stimulation was reported (33). Both of these cell communications are thought to occur by gap junctional transfer of secondary messenger molecules. These observations, together with other similarities between interferon and polypeptide hormones, led us to propose that there is a common cellular pathway of interferon and hormonal action. We have demonstrated that interferon can have hormonal activity (stimulation of myocardial cell beat frequency) and that hormonal (noradrenaline) stimulation

can result in interferon type antiviral activity. These findings have led us to conclude that interferon and hormonal action are probably mediated by common pathway(s). The cell-to-cell transmission of the reciprocal actions of interferon and noradrenaline not only gives further credence to a common pathway of their actions but also suggests that common transferred molecule(s) are generated after interaction of either substance with the appropriate cell membrane (34). Superficially, cyclic AMP seems a candidate for the interferon induced increase in beat frequency since cyclic AMP can cause this response (33) and interferon under certain conditions can elevate cyclic AMP levels (35). However, cyclic AMP alone cannot account for the antiviral effects since it is not antiviral (36) and interferon does not stimulate adenylyl cyclase in all cells (35). A more likely situation is that cyclic AMP and/or another small molecule(s) is responsible. This putative molecule may represent a new class of secondary messengers and thereby lead to elucidation of a new cellular control system. We suspect that a soluble substance extracted from interferon treated L cells may represent this molecule and be responsible for transfer of viral resistance and induction of the antiviral state. The transfer material has been found to be rapidly produced intracellularly after interaction of cells with interferon. It is highly unstable and disappears rapidly from cells. These are qualities we might expect of a secondary messenger molecule. Detection of the transfer material has been complicated by a control material, probably associated with the cell membrane, which is not induced by interferon, is released from sonicated cells and is antiviral. This material appears to be fibronectin. Fibronectin is a molecule of much current interest since it is in much higher levels in normal than transformed cells. Antiviral activity may provide a new function for this molecule and may be related to the transformed phenotype.

Two fundamental questions result from the hormonal studies. First, is interferon a hormone? The numerous similarities between interferon and polypeptide hormones indicate that interferon should be classified as such. These similarities coupled with our inability to distinguish interferon action from a hormonal response would seem to answer this question affirmatively. As such, the natural role of interferon may be regulatory with its effects on virus infections being secondary. The instances of low levels of interferon in normal individuals may not result from inapparent virus infections but may be reflective of this more general interferon regulatory mechanism. Additionally, this could be related to the side effects observed during clinical trials using high levels of interferon (37) as well as some aspects of viral pathogenesis.

A second important question is: What are the limits of responses to hormones? Classically, the actions of polypeptide hormones are well understood in terms of specific activation of their target tissues. The present findings suggest that there may also be patterns of different, hormonal responses induced by any one hormones and the pattern will vary with the cell type affected. For instance, hormones may not only have their known major action but may also protect tissues against viruses or maintain differentiation through interferon or other hormonal mechanisms. If this could be documented in vivo a new strategy of tissue targeted antiviral and antitumor therapy might evolve.

B. Methods of Procedure and Results

I. Transfer of interferon-induced viral resistance with fibroblastoid and epithelioid cells.

1. The kinetics of transfer of viral resistance within a population: We have previously shown that the rate and degree of interferon action is determined by the cell density and that the interferon response of cells is minimal when they are not in contact with one another. We interpreted this to be indicative of transfer of resistance within cell lines and cultures. By manipulation of the cell density we have timed the transfer of resistance within cell cultures. Confluent monolayers (1×10^5 cells/well) or isolated mouse L cells (1×10^4 cells/well) in Falcon Micro Test II tissue culture plates were treated briefly with their homologous interferon. At various times after interferon removal the cells were trypsinized and replated either as monolayers or isolated cells and infected with VSV. Twenty-four hours later the virus yield were compared to the appropriate control treated with culture media to determine the extent of interferon action. With cells that were initially monolayers, interferon activity increased as trypsinization and isolation of cells was delayed (Table 1). In other words, the earlier the cells were separated the less chance they had for transfer. There was 4 hrs from the beginning of the rise in interferon activity until the maximum was reached. This indicates that transfer occurs for 4 hrs within a cell culture. Contrarywise, initially isolated cells showed higher interferon activity the earlier they were trypsinized and concentrated into a monolayer (until 4 hrs when monolayer formation did not lead to an increase in interferon activity (Table 2). This time interval corresponds to the period of availability of the transfer material. It also closely approximated the time of transfer observed when monolayers are separated into single cells.

II. Production of human leukocyte interferon by non-sensitized human leukocytes co-cultured with "foreign" cells.

Since production of IFN- α (leukocyte interferon) is the first step in the transfer of viral resistance from non-sensitized leukocytes to co-cultured heterologous cells we conducted an indepth study of this process.

1. Characterization of inducer cells

a) Cells from different species: We have (27) demonstrated that mouse L cells and secondary mouse embryo fibroblasts were excellent leukocyte interferon inducers. Primary chick embryo and human WISH cells induced lower amounts of interferon. In order to maximize interferon induction, several other types of cells were tested for inducing efficacy. Induction and assay of foreign cell induced interferon was as follows: Human peripheral lymphocyte suspensions ($5-10 \times 10^6$ cells/ml) are prepared on a Ficoll-Hypaque gradient as previously described (27). One-tenth milliliter of the lymphocyte suspension in RPMI 1640 with 10% fetal calf serum is placed onto confluent cultures (about 10^5 cells/well) of each cell type in Micro Test II tissue culture plates (Falcon Plastics, Oxnard, Calif.). After 24-hr incubation in 4% CO₂ at 37°C the culture fluids were

harvested, clarified by centrifugation, and assayed for interferon as previously described (27). The interferon titer is expressed as the reciprocal of the dilution that inhibited 50% of the viral cytopathic effect. Interferon units are expressed in terms of the NIH human reference interferon. Table 3 shows differences in inducing abilities did not correlate with the species' relative evolution and confirm that B and not T cells are responsible for interferon production.

b) Erythrocytes: Erythrocytes were tested and found unable to induce interferon in nonsensitized lymphocytes (Table 4). Thus, erythrocytes apparently lack the IFN- α inducing factor and may serve as excellent controls for the purification of the IFN- α inducer from other cell types.

Similar experiments with murine lymphocytes were performed to determine if the process extended to the mouse. Xenogeneic, allogeneic, and allogeneic tumor cells were tested for their ability to induce interferon in nonsensitized mouse lymphocytes. As in the human system, xenogeneic and allogeneic tumor cells induced mouse interferon (Table 5).

2. Effect of inducer cell surface modifications on interferon. Cell contact is required between inducer cells and effector lymphocytes for interferon induction (27). Reaction kinetics plus the failure of soluble factors in the medium to induce interferon suggested that the inducing factor and receptors are present on the cell's surface membrane. To determine the nature of the inducing factor, specific enzymatic treatments were used to alter the cell membrane and these cells were assayed for their ability to induce interferon.

a) Proteases: Treatment of inducer cells with trypsin or pepsin destroyed their ability to induce IFN- α production by non-sensitized lymphocytes (Table 6). Human diploid fibroblasts did not induce IFN- α either before or after protease treatment. Thus it appears that the inducing factor is a cell surface protein.

b) Glycoproteins: Many components of cell surfaces are glycoproteins which can be involved in such processes as cell recognition, immunity, and malignant transformation. Likewise, inducing factor is probably a glycoprotein, since neuraminidase treatment destroyed the ability of cells to induce IFN- α production (Table 7).

3. Nonsensitized lymphocyte receptor for IFN- α induction: An assay for this receptor was developed and is based on inhibition of interferon induction by the receptor material. The receptor is removed from nonsensitized mouse lymphocytes by sonication. Soluble inducer (Table 8) are treated with the receptor material, and then mixed with nonsensitized lymphocytes, incubated, and the supernatant assayed for interferon. A reduction in IFN- α production was observed which is dependent on the relative concentrations of the receptor and inducer. We assume that the lymphocyte receptor complexes with the inducer and prevents inducer interaction with nonsensitized lymphocytes. The receptor

was also present on human lymphocytes (Table 9). As expected, sonicates from normal fibroblasts (which should not contain the receptor) failed to block induction of interferon (Table 10). Preliminary characterization indicates that the lymphocyte receptor is a cell surface protein since it is inactivated by trypsin treatment of lymphocytes (Table 11).

III. Transfer of interferon-induced viral resistance with lymphoid cells.

1. Cell type(s) that transfers interferon-induced viral resistance: Our laboratories have employed ficoll-hypaque gradients cell adherence, treatment with anti-immunoglobulin antibody and complement, and sheep red blood cell rosetting to show that the non-sensitized leukocyte which produces interferon in response to foreign cells is a B lymphocyte (28). These same procedures were used to separate cells of the immune system. Each of these cell types were assessed as to their ability to transfer viral resistance to heterologous cells. The demonstration of transfer followed the same techniques described for leukocyte interferon production (26 and Progress Report II.1.) except that co-cultures were challenged with VSV. Since VSV replicates poorly in leukocytes any virus yielded is from the heterologous cells. We found that both B and T lymphocytes will cause inhibition of virus yield from heterologous cells. Since we have previously shown that only B lymphocytes produce interferon when co-cultured with heterologous cells, exogenous human interferon had to be added to the T but not B cells to observe the transfer (Table 12).

2. Cellular sequence(s) of events in leukocytes and recipient cells during transfer:

a) Does IFN- α initiate transfer of viral resistance from leukocytes to recipient cells? Based on the close temporal relationship between production of interferon by leukocytes and development of resistance in L cells in co-cultures it appeared that interferon initiated the transfer of resistance. This idea was strengthened by the lack of transfer to cells which do not induce interferon production by leukocytes. To definitively establish that the IFN- α initiated the transfer process the following experiment was done. Human leukocytes were co-cultured with mouse L cells in the presence of anti-human IFN- α antisera. Since interferon must be externalized from a producing cell prior to acting, the antisera blocked the action of the human interferon on the human leukocyte. This in turn prevented the transfer of resistance from the human leukocyte to the L cell (Table 13). These data firmly establish that transfer of resistance is initiated by the interferon which is produced.

3. RNA synthesis in the transfer process: The antibiotic, actinomycin D, has been extensively employed to explore cellular events, such as interferon action, (4,5) which require RNA synthesis. We have shown that this compound will inhibit the transferred resistance (6,16). This data indicated that a transcriptional event is required in the recipient cell for the demonstration of transferred resistance. To determine if the leukocyte transfer of resistance was like that with fibroblastoid and epithelioid cells, similar experiments were done with leukocytes. We found that actinomycin D treatment of recipient cells blocked the development of resistance transferred from leukocytes (Table 14). A transcriptional requirement by the recipient cell

supports our hypothesis that the actual inducer of the antiviral protein gene is transferred and shows that transferred resistance is not mediated by the human antiviral protein or its mRNA since the action of either of these molecules should not require transcription. Also shown in Table 14 is that, although viral resistance is inhibited in actinomycin D treated L cells, there is the same amount of ^{51}Cr released from these cells. This shows that the inhibition of virus growth is not mediated by cell-mediated cytotoxicity.

IV. Transfer of interferon-induced immunosuppressive activity with lymphoid cells.

1. Cell type(s) that produces the interferon-induced suppressor factor:

We have shown that lymphoid cells transfer interferon's immunosuppressive activity. The indirect immunosuppressive action of interferon was found to be through induction of suppressor cells which in turn produce a suppressor factor that inhibits antibody production. Depletion of macrophages from the suppressor cell preparation by glass bead-glass wool columns did not affect the suppressor cell activity (Table 15), which suggests that the suppressor cell is a lymphoid cell. Preliminary treatment of suppressor cells with anti-Thy-1 serum to remove T-cell activity and with rabbit anti-mouse immunoglobulin serum to remove B cells with surface immunoglobulins did not affect the suppressor cell activity. Thus, the suppressor cell may be a null cell.

V. Hormonal action of interferon and antiviral action of polypeptide hormones.

1. Functional Relationship:

We have shown that interferon caused a species-specific hormonal response (noradrenaline-like stimulation of the beat frequency of cultured mouse myocardial cells). Noradrenaline induced an interferon-like antiviral state in mouse myocardial cells but not human amnion (WISH) cells. In conditions which demonstrate interferon-induced transfer of viral resistance, exposure of co-cultures of mouse myocardial cells and WISH cells to either human interferon or noradrenaline caused an increased beat frequency in the myocardial cells and development of antiviral activity in WISH cells, respectively. These studies strongly suggested common pathways of interferon and hormonal stimulation that are transmissible between cells. In order to determine whether this is a general phenomenon, we studied other possible in vitro hormonal actions of interferon and the potential antiviral activity of polypeptide hormones.

As has been previously shown (38-40) ACTH activity can be measured on mouse Y-1 adrenal tumor cells by a cell rounding assay (Fig. 1 left). Mouse, but not human interferon, also caused a dose dependent increase in Y-1 cell rounding. Similarly, ACTH and mouse interferon caused increased steroid production, while human interferon did not (data not shown). Thus, interferon can cause a species specific hormonal response on cells derived from the adrenal glands. Interferon caused development of a species specific antiviral state in mouse Y-1 adrenal tumor cells. ACTH also had antiviral activity on Y-1 cells (Fig. 1 right). However, ACTH did not have antiviral activity on mouse L cells or human amnion (WISH) cells (data not shown). These data show that ACTH has cell specific antiviral activity.

ACTH is composed of 39 amino acids, the first 13 of which are identical to α -melanocyte stimulating hormone (MSH). For this reason, ACTH can cause melanin production by melanoma cells (Fig. 2 left). Human, but not mouse interferon, also caused melanin production by human melanoma cells. We have tested two other human melanoma lines of which only one produced melanin in response to ACTH. Human interferon induced melanin in this same cell line without causing this response in the other. Human melanoma cells also became resistant to virus infection after ACTH or human (but not mouse) interferon treatment (Fig. 2 right).

While the ACTH used in these studies was highly purified, the interferon was a crude preparation. Thus it was necessary to determine whether the hormonal effects were actually due to interferon. Table 16 shows that specific rabbit antisera to mouse (C-243) and human fibroblast interferons neutralized mouse interferon induction of Y-1 cell rounding and human fibroblast interferon induction of melanin, respectively. These results provide strong evidence that the hormonal effects were due to the interferon in the preparations.

2. Structural Relationship:

The above similarities in action led us to propose that there might be structural similarities or identities between particular interferon types and certain polypeptide hormones. Initial immunologic studies supported this hypothesis by showing strong antigenic relatedness between human leukocyte (HuIFN α), but not fibroblast interferon (HuIFN β), ACTH and endorphins (41).

Fig. 3 (left panel) shows that anti-ACTH α (1-13) antiserum, in a dose dependent fashion, neutralized human leukocyte but not fibroblast interferon. Since human leukocyte and fibroblast interferons are known to differ antigenically as well as structurally, these data showed that the neutralization of leukocyte interferon by anti-ACTH (1-13) serum was specific. Increasing concentrations of ACTH blocked the neutralization of human leukocyte interferon by anti-ACTH α (1-13) serum (Fig. 3, right panel). Thus, the antiserum was recognizing ACTH antigenic determinants on the human leukocyte interferon molecule. The finding of almost complete blockage by ACTH indicated that the antiserum was not recognizing determinants in leukocyte interferon other than ACTH. It should be noted that this is a highly specific rabbit antiserum which was raised against synthetic ACTH α (1-13) (also melanocyte stimulating hormone, α -MSH). It has a titer by radioimmunoassay of 1:38,000 and 1:1000 against ACTH α (1-13) and natural porcine ACTH (1-39), respectively (Bio-Ria, Brussels, Belgium).

It is known that ACTH and endorphins (brain polypeptide hormones with opiate activity) can be derived from a single common precursor molecule that is immunoprecipitable by specific antisera to either ACTH or endorphins (42). Leukocyte interferon also appears to contain an endorphin-like antigenic structure, because a highly specific rabbit anti-endorphin serum (raised against synthetic γ -endorphin, Bio-Ria, Brussels, Belgium) neutralized human leukocyte interferon activity (Table 17). This neutralization was blocked by γ -endorphin. Antisera directed against polypeptide hormones that are structurally unrelated to ACTH and endorphins did not neutralize leukocyte interferon. Specifically, there was no neutralization with anti-human LH (leuteinizing hormones) (1:80) or FSH (follicle stimulating hormone) (1:300) sera (gifts of Dr. E.R. Smith). Taken together these

data showed that human leukocyte interferon shares antigenic determinants with ACTH and the endorphins.

Further evidence that interferon and ACTH have common antigenic sites is the finding that an antiserum to human leukocyte interferon (Table 18) or anti-ACTH α (1-13) serum (data not shown) neutralized the bioactivity of ACTH. Proof that human leukocyte interferon antiserum recognized an ACTH determinant in the leukocyte interferon molecule was found in the observation that ACTH blocked the neutralization of leukocyte interferon by anti-leukocyte interferon serum (Table 19). This blockage was incomplete, which suggested that leukocyte interferon antiserum also recognized determinants other than ACTH on the interferon molecule. These common antigenicities of ACTH and leukocyte interferon may have important practical applications for the purification and immunodetection of leukocyte interferon. In fact, anti-ACTH antibody affinity chromatography has been used to purify leukocyte interferon 10,000 fold in a single step (see below).

Table 20 shows data which suggests that structural similarities underlie the antigenic relatedness of human leukocyte interferon and ACTH. All of the bioactivity of ACTH resides in the amino terminal 24 amino acids [ACTH α (1-24)] of this molecule. ACTH α (1-24) is common to all species, pepsin insensitive and has a molecular weight of 2400. Preliminary experiments had shown that human leukocyte interferon lacks ACTH activity on mouse Y-1 adrenal tumor cells. We reasoned that if the ACTH α (1-24) sequence was found within human leukocyte interferon, pepsin digestion of leukocyte interferon should generate ACTH activity. ACTH activity was measured by an increase in the number of rounded Y-1 cells when ACTH is present.

Pepsin digestion of human leukocyte interferon completely destroyed its antiviral activity and caused the appearance of ACTH activity (Table 20). The pepsin cleaved fragment was neutralized by anti-ACTH α (1-13) antiserum and had a molecular weight greater than 1000 but less than 5000 as determined by Amicon ultrafiltration. Human fibroblast interferon had no ACTH activity either before or after pepsin treatment. The high specific activity of leukocyte interferon ($10^{8.5}$ U/mg protein) raises the question: Can enough ACTH be generated from an interferon preparation for detection in the Y-1 cell assay? Based on its specific activity, 1000 U of leukocyte interferon should contain approximately 3 ng of interferon. Total cleavage of 1000 U of leukocyte interferon theoretically should yield 0.6 ng of ACTH since the molecular weight of ACTH is approximately 1/5 that of leukocyte interferon. This amount of ACTH is easily detectable in the Y-1 cell rounding assay (see Table 2). In fact, the amount of ACTH activity generated by pepsin treatment of 1000 U of leukocyte interferon approximates that seen with 10^{-4} units of ACTH (about 0.5 ng) when parallel titrations were done. These data strongly suggest, based on pepsin insensitivity and the molecular weight of the ACTH-like fragment of human leukocyte interferon, that the antigenic relatedness of leukocyte interferon and ACTH are based on structural similarities or identities. Table 21 shows that the ACTH activity in pepsin treated interferon preparations is neutralized by anti-ACTH α (1-13) serum and is not present in fluids from non-interferon producing lymphocyte cultures. Thus the bioactivity is immunologically related to ACTH.

As previously mentioned, endorphins and ACTH are derived from a common precursor molecule which is immunoprecipitable by both antisera to ACTH and endorphins. Likewise, the antiviral activity of human leukocyte interferon preparations was neutralized by specific anti- γ endorphin as well as anti-ACTH α (1-13) sera. Endorphins bind to specific receptors on brain tissue and compete with

opiates for these binding sites (for review see 43). A human leukocyte interferon preparation was assayed for endorphin-related substances by an assay measuring inhibition of ^3H -dihydromorphine binding to mouse brain tissue (Table 22). A significant amount of dihydromorphine binding was inhibited by the interferon, but not by a control preparation. The inhibition was dose dependent and was on the order of that observed with 10^{-7} to 10^{-8} γ -endorphin. The above data demonstrate the bioactivities in leukocyte interferon which are expected based on previous immunological evidence.

Although the above immunologic data show a relationship between leukocyte interferon, ACTH, and endorphins, they did not prove the molecules are covalently linked. Purified human leukocyte interferon was examined by polyacrylamide gel electrophoresis to determine if the ACTH and endorphin activities were inseparable and if interferon was a common precursor.

Interferon was purified to 10^7 U/mg protein by anti-ACTH α (1-13) antibody affinity chromatography which exploited the antigenic relatedness between human leukocyte interferon and ACTH. A highly specific antibody made against a synthetic fragment of ACTH (ACTH α 1-13) was purified from commercially available antisera (Bio Ria) on an ACTH-sepharose affinity column (Fig. 4). This purified ACTH α (1-13) antibody was then coupled to cyanogen bromide-activated sepharose (4 ml). Figure 5 shows that when 4.5×10^5 units of human leukocyte interferon were loaded onto the ACTH α (1-13) antibody-sepharose affinity column 4.5×10^4 units (10%) did not bind while 4.0×10^5 units (90%) bound and was eluted by 0.1 M glycine buffer (pH2). The 10% non-bound interferon failed to bind when applied a second time which indicates a different species of interferon rather than saturation of the column. The specific activities of the applied and bound leukocyte interferon were 10^5 and 10^7 units/mg protein, respectively. Thus this 4 ml antibody affinity column can purify human leukocyte interferon 10,000 fold in a single step with 90% recovery of the applied activity. We have determined that this antibody affinity column has a capacity to bind 5 μg of ACTH. Assuming an equal affinity of the antibody for ACTH (molecular weight of 4000) and leukocyte interferon (molecular weight of about 20,000) (which seems reasonable based on relative neutralization titers) and a known molecular weight ratio for these molecules of 5, the 4 ml column should hold 25 μg of leukocyte interferon (about 10^7 units). This purification procedure did not overly bias the results since 90% of the interferon activity was bound to the column. The 10% of the interferon population not bound to the column did not contain ACTH activity when assayed on Y-1 cells either before or after pepsin treatment (data not shown). Elution of the interferon from the column with pH 2 buffer generated ACTH activity which previously was not present. This was not totally unexpected since acid cleavage of ACTH was previously reported (44).

Electrophoresis of the purified human leukocyte interferon confirmed the presence of at least two molecular species of interferon (Figure 6a) (45). Two peaks of interferon activity were detected at apparent molecular weights of 23,000 (23K) and 18,500 (18.5K) daltons. Assay of the gel slices on Y-1 cells showed ACTH activity present in a low molecular weight form co-migrating with a known sample of ACTH. When the material from the gel in Figure 6a was digested with pepsin and assayed on Y-1 cells, ACTH activity was generated from the material associated with the larger molecular weight interferon (Figure 6b). ACTH activity was still associated with the material at the bottom of the gel and thus was pepsin insensitive. No activity could be generated from the lower molecular weight interferon peak. Though not directly determinable because of the minute amounts of

protein added, the interferon bands in the polyacrylamide gel should be essentially homogenous. Based on the experience of others (45) utilizing very similar techniques and interferon of similar purity, SDS polyacrylamide gel electrophoresis resulted in a 20 fold purification. Since our starting material was 10^7 U/mg protein, the interferon in the gels should have a theoretical specific activity of $10^{8.3}$ U/mg protein. In order to show that the low molecular weight ACTH activity could be derived from the 23,000 dalton leukocyte interferon, the eluted gel slice (23,000 dalton) was subjected to pepsin treatment, followed by electrophoresis (Figure 6c). No ACTH activity was present in gels of nondigested interferon. Pepsin digestion of interferon, however, generated a peak of low molecular weight ACTH activity. No interferon anti-viral activity remained following pepsin digestion. This data suggests that ACTH activity is derived from and covalently linked to the leukocyte interferon molecule. It is tempting to speculate that the large form of the interferon is inherently unstable and following protease or acid treatment (such as that used to elute the affinity column) causes cleavage of the ACTH fragments. This (pH 2 elution) would account for the presence of low molecular weight ACTH activity present in gels of affinity purified interferon. Interferon not subjected to low pH or protease has little or no low molecular weight ACTH activity (Figure 6c). Furthermore, it seems plausible that the 18,500 dalton interferon might be derived from the 23,000 dalton species since the difference in their molecular weights is approximately that of ACTH (about 4000 daltons).

ACTH migrated with the buffer front in the 12.5% polyacrylamide gels and, therefore, did not separate entirely on a molecular weight basis. To show that ACTH generated from interferon does co-migrate with natural ACTH, samples were electrophoresed in duplicate on 15% polyacrylamide gels (Figure 7). ACTH activity from interferon and the standard banded in the same region of the gels. Endorphin activity was detected in the gel slices associated with both the 23,000 and 18,500 dalton interferon bands plus several low molecular weight peaks of activity (data not shown). Following pepsin treatment of interferon, there appeared to be an increase in low molecular weight endorphin material.

Since leukocyte interferon is a lymphocyte product and is apparently a precursor to ACTH and endorphins, specific antisera to these hormones provided a means to study the lymphocytes responsible for interferon, ACTH and endorphin production. Interferon was induced by Newcastle Disease virus (NDV) infection and lymphocytes were assayed for ACTH and endorphins by an indirect immunofluorescence technique. Figure 8c shows that virtually all NDV infected (interferon producing), but not non-infected (Figure 8b), lymphocytes exhibit positive immunofluorescence with anti-ACTH α (1-13) serum at 18 hr post infection. There was a progressive increase in the percentage of positively staining lymphocytes with time, which paralleled the increase in leukocyte interferon. At 1, 6, and 8 hr post infection, 0, 30 and 50% of NDV-infected lymphocytes showed positive immunofluorescence, respectively. The maximum percentage (100%) of stained lymphocytes was observed between 18 and 24 hrs post infection and thereafter declined. Trypan blue dye exclusion indicated that greater than 95% of both infected and noninfected lymphocytes were viable at 24 hr post infection. Figure 8d shows that NDV infected lymphocytes also stained with anti-endorphin serum. Increases in the percentage of endorphin positive cells were similar to those seen for ACTH. Noninfected lymphocytes did not stain with this antiserum (not shown). The fluorescent staining pattern was cytoplasmic for both sera.

Both rabbit anti-ACTH α (1-13) and anti- γ -endorphin sera were prepared against synthetic antigens and are highly specific reagents. Table 23 demonstrates the specificity of the immunofluorescent staining. Only NDV infected lymphocytes stained with the anti-ACTH α (1-13) or anti- γ -endorphin sera. The staining reaction of the anti-ACTH α (1-13) sera was blocked by absorption with porcine ACTH (1-39) but not with NDV or noninfected lymphocytes. This shows that a viral product of NDV infection was not staining. All the lymphocytes inoculated with NDV appeared to be infected since 100% of the cells showed positive staining with anti-NDV serum. Immunofluorescence with anti-human immunoglobulin serum stained equal percentages (about 20%) of infected and noninfected lymphocytes suggesting that surface immunoglobulin or Fc receptors were not involved in the anti-hormone staining. Mouse pituitary tumor cells (AtT-20) which spontaneously produce ACTH (42) stained with anti-ACTH α (1-13) serum; whereas NDV infected human (WISH) amnion cells (which produce fibroblast but not leukocyte interferon) did not stain (data not shown). This result is further evidence that the anti-ACTH α (1-13) serum was not detecting NDV antigens and is specific for leukocyte interferon. Stimulation of human lymphocytes with several mitogens (staphylococcal enterotoxin A, concanavalin A, and phytohemagglutinin) failed to elicit positive immunofluorescence with anti-ACTH α (1-13) serum, which indicates that mitogenesis alone is not sufficient to induce the ACTH-like material.

Human tumor and xenogenic cells induce leukocyte interferon production by non-sensitized human lymphocytes. When transformed mouse (L-929) or human (WISH) cells were co-cultured with human lymphocytes, the lymphocytes, but not the tumor cells, stained with anti-ACTH α (1-13) serum and produced leukocyte interferon. In the presence of L and WISH cells, only 52 and 54% of the lymphocytes appeared to produce ACTH-like material, respectively (data not shown). Reasons for the difference between transformed cell and virus induction of ACTH-like substances are unknown at present, but may be related to differences in the mechanism and efficiency of induction or the lymphocyte types which are affected.

The finding that essentially 100% of NDV infected lymphocytes were stained with either anti-ACTH α (1-13) or anti- γ endorphin sera at 18 hr post infection is important for a number of reasons. First, a cross reaction between human immunoglobulin class IgG1, and β -endorphin and ACTH has been reported (46). It seems that this is not the reason for our findings since immunoglobulins are on the surface of only about 20% of peripheral lymphocytes (B cells) and IgG1 on about 1% (47). Further support for a negligible role for this crossreaction is the inability to generate ACTH activity from IgG1 by protease digestion while it is readily observed in pepsin-treated interferon. Second, the anti-ACTH α (1-13) and anti- γ -endorphin sera do not crossreact (data not shown) which implies that each lymphocyte can produce both antigens, presumably in the form of interferon. Lastly, apparently all lymphocyte types (B, T and null cells) in peripheral blood can produce ACTH, endorphin and leukocyte interferon in response to NDV. Thus, this response to virus infection seems to be of a generalized nature and may represent a mechanism for the detection of leukocyte interferon-producing cells.

VI. Cell-free transfer of interferon-induced viral resistance.

As previously indicated (Progress Report 2), our finding of an interferon-induced transfer material in extracts of interferon treated cells has been complicated by the presence of an antiviral substance in control cells (Control Material, CM). We felt that to more clearly understand the transfer material, we must first elucidate the nature of CM in the hope of eventually removing it as a variable.

1. Is CM fibronectin?

Several observations had indicated the possibility that CM might be fibronectin. Studies have shown that like fibronectin, CM is greater than 300,000 MW, extremely trypsin sensitive, produced by normal more than transformed cells, and probably released from the cell membrane. We have also shown that CM's activity is removed by a gelatin/sepharose affinity column which selectively binds fibronectin. To determine whether CM was fibronectin, we have employed specific antisera. We found that commercially available rabbit anti-human fibronectin antibody was able to neutralize CM (Table 24). CM from mouse embryo and human lung cell sonicates was neutralized but not CM from human WISH or mouse L 929 cells which are transformed cells. This may indicate the presence of structurally or antigenically different CM or fibronectin molecules. Further evidence that CM may be fibronectin is the finding that treatment of transformed L 929 cells with CM from normal human lung cells changed the morphology of the L cells. Similar to fibronectin, CM caused the L cells to assume a more "normal" morphology. This change could be blocked by antibody to fibronectin.

To determine whether fibronectin (CM) might be associated with interferon action, we examined the effect of interferon treatment on the levels of fibronectin in transformed cells. As shown in table 25, fibronectin as determined by indirect immunofluorescence increased in transformed human WISH cells treated with human interferon. To eliminate the possibility that the fluorescence was due to soluble fibronectin present in the human interferon preparation adsorbing to the WISH cells, interferon was subjected to gelatin/sepharose affinity chromatography to remove endogenous fibronectin. As shown in table 26, the increase in immunofluorescence remained after removal of soluble fibronectin. It should also be noted that mouse interferon did not cause an increase in immunofluorescence. This is further evidence that the increased fibronectin was not acquired from the interferon preparations and also shows that the increase is species specific (like other actions of interferon). Soon after this phenomenon was observed, a publication appeared indicating that interferon caused a rearrangement of fibronectin on cells (48). Preliminary evidence (Table 27) shows that interferon caused an increase in the amount of ³H-amino acids incorporated into a gelatin binding protein, presumably fibronectin.

This suggests that in addition to any rearrangement, there is an increase of fibronectin. Since interferon appeared to increase the cell associated levels of fibronectin, we wondered if this increase was related to the antiviral effect. Preliminarily, exposure of interferon treated human WISH cells to antifibronectin antibody resulted in a significant decrease in antiviral activity. In light of the data presented, there is a possibility that fibronectin might be the transfer material or may be induced by the transfer material. This is currently under study.

2. Characteristics of the antiviral action of CM.

a) Kinetics of action: CM produced in L cells was placed on varying cells for varying periods of time. Cultures were then washed and challenged with VSV. Twenty-four hours later virus yields were determined. Table 28 shows that with increasing times of treatment with CM, there was a progressive decrease in the yield of virus. Significant inhibition was first observed after 6 hrs incubation.

b) Protein synthesis: It appeared that CM might induce an antiviral state in cells since cells retain antiviral activity after CM is removed by washing. Data consistent with this are shown in Table 29. Cycloheximide treated or control cells were treated for the 8 hrs (2a) with CM. Cultures were washed to remove the cycloheximide and were challenged with VSV. Virus yields were determined 24 hrs later. We found that cycloheximide blocked CM's antiviral action. CM activity involves de novo synthesis of a protein since its action was blocked by cycloheximide.

C. Discussion and Conclusions:

IFN- α production is the first step in the transfer of viral resistance from non-sensitized lymphocytes to co-cultured heterologous cells. With the exception of erythrocytes, this process can be initiated by virtually any xenogeneic normal cell and any xenogeneic or allogeneic tumor cell. Thus it seems that the process may be a general type of mechanism by which B lymphocytes recognize foreignness. The inducer on the foreign cell is apparently a cell surface glycoprotein which is present on both normal (xenogeneic) and transformed cells. It is unknown at present whether this molecule(s) is the same or different on various species and types of cells. The IFN- α inducer seems to be recognized by a surface protein receptor on non-sensitized lymphocytes which is not present on normal fibroblasts. In a specific sense, an understanding of this inducer: receptor system should elucidate the initial mechanisms involved in the production of IFN- α and the subsequent initiation of the lymphocyte transfer process. Generally, it may represent an important mechanism of recognition of tumor and other foreign cells. In this regard, it will be important to determine whether specific recognition is involved with each foreign cell.

While B but not T lymphocytes appear to be the major IFN- α producer in response to foreign cells, both B and T lymphocytes can transfer viral resistance. The difference, of course, is that exogenous IFN must be added to T lymphocytes for transfer to occur. In a mixed population of lymphocytes, we assume that T lymphocytes are recruited to transfer viral resistance via IFN- α production by B lymphocytes.

Based on these and previous findings (26-28), we have developed the following operational model for the lymphocyte transfer process. Xenogeneic or allogeneic tumor cells are recognized by nonsensitized B lymphocytes and IFN- α is produced. This IFN acts back on B and T lymphocytes to generate a substance(s) which is transferred directly to the xenogeneic cells and causes induction of the antiviral state. Since the transfer process requires a transcriptional event in the recipient cell, the transferred material may represent the molecule which transmits IFN's signal from the cell membrane to the nucleus (6).

Taken together, these studies strongly suggest a new and efficient host immune defense against virus infection. In vivo, the system would be activated by IFN (produced by B lymphocytes in response to virus and virus-infected cells or by virus-infected cells alone). Once activated, migrating lymphocytes could transfer resistance to other tissues. Unlike cytotoxic cells, cells capable of transferring resistance could rapidly help protect uninfected and recently infected cells without destroying them. In fact, in the systems herein reported, protection of virus-infected cells correlated with transfer of resistance but not with cytotoxicity by lymphocytes.

Unlike the transfer of viral resistance, the transfer of IFN-induced immunosuppression is not mediated by B and T lymphocytes. Although depletion of macrophages did not affect the suppressor cell activity (Table 4), which suggests that the suppressor cell is a lymphoid cell. Preliminary treatment of suppressor cells with anti-Thy-1 serum to remove T-cell activity and with

rabbit anti-mouse immunoglobulin serum to remove B cells with surface immunoglobulins did not affect the suppressor cell activity. Thus, the suppressor cell may be a null cell.

In addition to the above novel effects on immune cell function, the literature is replete with many other diverse actions of interferon (for review see 49). These actions are usually thought of in terms of a unique mode of action of interferon. However, one wonders how these many effects are mediated via a unique pathway. We have proposed that the action of interferon is not unique but shared with other hormones. While specifically targeted hormones may be limited in the number of effects they have by the number of different target cells or tissues with complementary receptors, no such barrier seems to exist for interferon. Most nucleated cells appear sensitive to interferon. Since interferon and hormones apparently share common pathways, interferon should cause many responses which are dictated by the cell type or tissue. Data supportive of this idea are the present findings of interferon induction of steroidogenesis and melanogenesis. These additional hormonal actions of interferon lead one to further question whether many of the biochemical changes in interferon treated cells are really interferon specific or more generally of a hormonal nature. The present studies raise to three the number of cell types with specialized functions which can be invoked by interferon, i.e., mouse myocardial cells, adrenal tumor cells and melanoma cells. We expect that interferon may have as many actions as there are specialized cells on which to measure them. Conversely, these studies have shown that like noradrenaline another hormone, ACTH, can cause antiviral activity on its target cell types. This antiviral state was cell but not species specific. These findings support the view that beyond the point of the membrane receptor, interferon and polypeptide or polypeptide-like hormones share pathways leading to cell activation. If this is true then perhaps the natural function of interferon is hormonal and that of hormones includes protection of tissues against viruses.

Further evidence for this is the finding of antigenic and structural similarities between leukocyte interferon and ACTH and γ -endorphin which implies that leukocyte interferon may be a precursor to these hormones. In vivo proteolytic cleavage of leukocyte interferon could generate peptide hormones (ACTH and endorphins) which influence the neuroendocrine system. The result would be that leukocyte interferon may play a pivotal regulatory role via known hormonal circuits. This idea is perhaps even more appealing in light of the expanding list of leukocyte interferon inducers, i.e. viruses, bacteria and bacterial products, double stranded RNA, tumor cells, xenogeneic cells, etc. The relationship we have observed between leukocyte interferon, ACTH, and endorphins, raises the interesting possibility that different interferon types or other lymphocyte products (lymphokines) may be related to known peptide hormones or that they derive from a common molecule(s). If in vivo cleavage occurs, the described effects of interferon on cancer, hepatitis, herpes and common colds, etc. may require a reevaluation which considers possible secondary hormonal effects. Furthermore, leukocyte interferon production and subsequent modification might explain the pathophysiology of certain diseases.

Since these studies concerned a product of lymphoid cells, the data presented here suggest that the immune system can produce a precursor to neuroendocrine hormones. In fact, leukocyte interferon-producing lymphocytes showed positive fluorescence when stained with either ACTH $_{1-13}$ or endorphin

antisera in an indirect immunofluorescence procedure. Thus direct and known polypeptide hormonal circuits appear to exist between the immune system and the neuroendocrine system. This circuit may represent a mechanism by which the immune system signals other tissues, such as the brain and adrenal glands, during infection, tumor formation and chemical insult. An understanding of this putative lymphoid-pituitary-adrenal axis and the involvement of interferon may lead to new strategies for the detection and treatment of tumors, autoimmune and infectious diseases.

Progress towards elucidation of the putative secondary messenger molecule(s) thought to be responsible for the transfer of interferon's hormonal and antiviral actions was hampered by the presence of an antiviral substance(s) in control and interferon treated cells (Control Material, CM). The CM has tentatively been identified as fibronectin. Interestingly, the antiviral activity of CM is similar to interferon in that it requires induction of a new protein. Furthermore, interferon seems to induce fibronectin production. Thus it is tempting to speculate that fibronectin may be involved in the antiviral action of interferon. Preliminary evidence for this is the inhibition of interferon action by anti-fibronectin serum. If fibronectin is involved in interferon action, it may be the transfer material and/or may be induced by the transfer material. Further study of these possibilities should delineate a new aspect of interferon's antiviral action, especially as related to the interferon induced transfer processes. Additionally, they may define new functions for fibronectin, a molecule of much current interest because of its prevalence on normal as opposed to transformed cells.

Table 1. Effect of Redistribution of L Cells from a Monolayer to Single Cells on Interferon Action

Manipulation*	Log ₁₀ Inhibition of VSV Yield at:				
	0 hr	1 hr	2 hr	4 hr	6 hr
Single cells to single cells	none	none	0.1	0.2	0.5
Monolayer to single cells	none	0.3	0.7	1.5	1.6

*Isolated single cells (1×10^4 L cells/well) or monolayers (1×10^5 L cells/well) in microtiter plates were treated for the indicated times with 10 U/ml of mouse interferon, trypsinized and replated as single cells (1×10^4 L cells/well). Cells were then infected with VSV and the yield of virus determined 24 hrs later by a microplaque assay (6).

Table 2. Effect of Redistribution of L cells from Single Cells to Monolayer on Interferon Action.

Manipulation*	Log Inhibition of VSV Yield at:			
	0 hr	2 hr	4 hr	6 hr
Single cells to monolayer	none	0.3	0.9	0.8
Single cells to single cells	none	0.2	0.4	0.2

*Single cell suspension (1×10^5 L cells/ml) were treated for the indicated times with 10U/ml of mouse interferon washed and cells were either plated (0.1 ml/well) at 1×10^6 cells/well (single cells) or concentrated by centrifugation (1×10^6 cells/ml) and plated (0.1ml/well) at 1×10^5 cells/well (monolayer). Cells were then infected with VSV and the yield of virus determined 24 hrs later by a microplaque assay (6).

Table 3

Induction of interferon in mixed cultures containing
human T and B cells and xenogeneic or
allogeneic cells

Cell line	Animal origin	Human Interferon units/ml ^a	
		T	B
WIRA	Rat	10	3,000
RK-13	Rabbit	3	3,000
Glioma ^b	Human	0	10,000
Neuroblastoma	Human	0	10,000
WISH	Human	3	10,000

^a Antiviral units after 24 h incubation of mixed cultures of monolayers of the cell line with fractionated lymphocytes.

^b Glioma and neuroblastoma cells were obtained from Dr. E. Tiffany-Castiglioni, University Texas Medical Branch, Galveston, Texas.

Table 4

Failure of heterologous erythrocytes to induce leukocyte interferon
in nonsensitized human lymphocytes

Inducing Cell	Concentration (no/0.1 ml)	Interferon (units/ml)
Human RBC	1×10^5	0
	5×10^5	0
Sheep RBC	1×10^5	0
	5×10^5	0
Mouse Embryo Fibroblast	1×10^5	300
	5×10^5	1000
None	-	0

Lymphocytes were prepared and co-cultured as described in text. Human erythrocytes (RBC) were collected from the pellet of Ficoll gradient separated whole blood. The human erythrocytes and lymphocytes were from different individuals. The sheep RBC was a commercial preparation in Alsever's solution (Colorado Serum Co. Labs., Denver, Colorado).

Table 5

Interferon induction in nonsensitized mouse spleen cells
and human lymphocytes co-cultured with heterologous cells

Lymphocyte (1×10^6)	Inducing Cell (1×10^6)	Interferon (U/ml) content when assayed on:	
		Human WISH cells	Mouse L cells
Human	Mouse L	3000	<3
	Human fibroblast	<3	ND
	None	<3	ND
Mouse spleen	Human WISH	<3	1000*
	Mouse embryo	ND	<30
	None	ND	<3

*Mouse spleen interferon was greater than 90% stable to pH 2 treatment whereas 100% of mouse immune interferon was inactivated by pH 2 treatment. Human lymphocytes were prepared and co-cultivated with foreign cells in microtiter plates (0.2 ml total volume/well) as described in text. Mouse spleens were removed from C57 Bl/6 mice, then teased and pipetted to separate into individual cells. Spleen cells were co-cultured with human amnion (WISH) cells under identical conditions as the human lymphocytes. Mouse and human interferons were assayed on mouse L or human WISH cells with a micro plaque-reduction technique with vesicular stomatitis virus (VSV).

Table 6

Effect of protease treatment of inducer cells on interferon
induction in nonsensitized mouse spleen cells

Enzyme Treatment	Concentration (ug/ml)	Length of Treatment (min)			
		10	30	60	120
None	0	300*	300	300	300
Trypsin	10	300	300	100	30
	100	300	100	60	30
	1000	<30	<30	<30	<30
Pepsin	10	300	300	300	100
	100	100	100	100	30

*Interferon (U/ml)

Mouse spleen cells were prepared, diluted (1×10^7 /ml) and co-cultivated as described in text. WISH cells (1×10^6 /ml) were diluted into EMEM containing no serum and treated with trypsin (302 U/mg; Millipore Corp., Freehold, N.J.) and pepsin (3180 U/mg; Sigma Chem. Co., St. Louis, MO) at the indicated concentrations or length of time.

Table 7

Effect of neuraminidase treatment of inducer cells on the induction of interferon in nonsensitized mouse spleen cells

Enzyme Treatment	Concentration (ug/ml)	Length of Treatment (min)			
		10	30	60	120
None	0	160 ^a	160	160	160
Trypsin	100	160	40	40	20
Neuraminidase ^b	0.002	80	80	80	80
	0.026	80	80	40	40
	0.256	40	40	40	20

^a Interferon (U/ml)

^b 1 U/25.6 μ g (Sigma)

Table 8. Mouse Lymphocyte Receptor for IFN Inducer.

Inducer	Receptor (dilution)	Sonication (minutes)	Interferon (U/ml)
+	-	-	100
+	Undilute	0	60
+	1:10	0	100
+	Undilute	1	<30
+	1:10	1	60
+	Undilute	5	<10
+	1:10	5	<30
-	Undilute	1	0
-	-	-	0

Inducer was the supernate fluids of sonicated (1 min) and clarified human WISH cells. Receptor was prepared by sonication of C57/B1 mouse spleen cells (2.5×10^6 cells) for the indicated times followed by centrifugation to remove cell debris. Undiluted inducer was mixed with the indicated amounts of receptor and incubated for 45 minutes at 37°C. Mixtures are then added (0.1 ml) to 5×10^6 mouse spleen cells (0.1 ml) in microtiter plates. After 24 hrs incubation at 37°C in 4% CO₂, supernatant fluids are assayed for mouse interferon by microplaque reduction on mouse L cells.

Table 9. Human Lymphocyte Receptor for IFN Inducer.

Inducer (undilute)	Receptor (undilute)	Interferon (% of Control)
+	+	30
-	+	0

Procedures are the same as in Table 8 except mouse L cells were the source of inducer and human lymphocytes (sonicated 1 minute) was the source of receptor. Receptor above did not induce interferon.

Table 10. Lack of a Receptor on Mouse Embryo Fibroblasts (MEF) for the IFN Inducer.

Inducer (undilute)	Receptor (undilute)		Interferon (% of Control)
	MEF	Mouse Spleen Cell	
+		+	30
+	+		100
-	+		0
-		+	0

Procedures are as described in Table 8 except that receptor material was also made from mouse embryo fibroblasts. Mouse embryo fibroblasts and receptor do not induce interferon production by mouse spleen cells.

Table 11. Trypsin Sensitivity of Mouse Lymphocyte Receptor for IFN Inducer

Spleen cells/0.1 ml	Treated		Interferon (U/ml)
	Minutes	Trypsin (mg/ml)	
1 x 10 ⁶	0	0	50
"	5	1	40
"	15	1	<10
"	30	1	<10
"	60	1	<10
5 x 10 ⁶	0	0	120
"	5	1	90
"	15	1	60
"	30	1	<10

C57/B1 mouse spleen cells were treated for the indicated times with trypsin. Spleen cells were washed and added to human WISH cells (1 x 10⁵) in microtiter plates. After 24 hrs supernatant fluids were assayed for mouse interferon.

Table 12. Transfer of interferon-induced viral resistance by B and T lymphocytes

Lymphocytes	Interferon (U/ml)				
	Log ₁₀ Inhibition of Sindbis Virus Yield				
	0	1	10	100	1000
B	1.5	-*	-	-	-
T	none	0.3	0.8	1.1	2.0

Human T or B lymphocytes were treated with the indicated concentrations of human IFN- α and co-cultured at a 10:1 ratio with mouse L cells. Twenty four hours later co-cultures were challenged with Sindbis virus. After overnight incubation virus yields were determined. Human IFN- α was present in the supernatant fluids of non-interferon treated B but not T cells.

Table 13 Inhibition of leukocyte transfer of antiviral activity by anti-IF antiserum

Culture condition*	Human IF (U/ml)	Inhibition of virus (%)
Control	100	91
Normal rabbit serum	100	89
Rabbit anti-human leukocyte IF serum	10	10

* Mouse L cells were cocultured with human leukocytes and rabbit antiserum (National Institute of Allergy and Infectious Diseases, Bethesda, Md.) to human leukocyte IF. The leukocyte-to-recipient cell ratio was 5:1. After 16 h of incubation, fluids were harvested for IF assay, and the cultures were challenged with Sindbis virus (multiplicity of infection = 50). Virus was harvested 24 h later.

TABLE 14 Effect of actinomycin D treatment of L cells on development of viral resistance transferred from human leukocytes during cocultivation

Cultures*	Time after addition of leukocytes (h)	Human IF (U/ml)	Virus inhibition (log ₂)	⁵¹ Cr release (%)
Leukocytes plus L cells	4	300	1.5	3
	16	3,000	2.0	47
Leukocytes plus L cells treated with actinomycin D	4	300	0.3	3
	16	1,000	0.6	50

* Mouse L cells were pretreated with ⁵¹Cr for 4 h and 5 µg of actinomycin D per ml for 2 h before addition of leukocytes to recipient cells at a ratio of 10:1. IF levels, virus inhibition, and percent specific ⁵¹Cr release were determined at the indicated times.

Table 15. Effect of macrophage depletion on immunosuppressive effect of interferon-treated spleen cells*

Macrophage depletion	Interferon treatment	Viable cells per ml added to cultures	Anti-sheep erythrocyte PFC per culture ± SD*	Inhibition (%)
Not depleted	Treated	8 × 10 ⁶	1,200 ± 368	88
	Untreated	8 × 10 ⁶	9,860 ± 764	
	Treated	4 × 10 ⁶	2,400 ± 368	62
	Untreated	4 × 10 ⁶	6,320 ± 735	
Depleted	Treated	8 × 10 ⁶	530 ± 184	93
	Untreated	8 × 10 ⁶	7,560 ± 113	
	Treated	4 × 10 ⁶	1,640 ± 820	71
	Untreated	4 × 10 ⁶	5,640 ± 113	

* Suppressor cells were induced with interferon as described in Table 1, footnote a (1,000 U, 24 h). Macrophages were depleted by passing the cells through a glass wool-glass bead column. The macrophage-depleted cells were washed twice before addition to syngeneic cultures at the indicated concentrations.

* SD, Standard deviation.

Table 16. Neutralization of the hormonal activity of Interferon by anti-Interferon sera.

Sample ^a	Activity Melanin ($\mu\text{g/ml}$ + S.D.)	Rounded Y-1 cells/ Microscopic Field + S.D.
Human Interferon (150 U/ml) ^b	1.8 + 0.1	-
Human Interferon (150 U/ml) + Anti-human Interferon Serum (1:60)	0.5 + 0.1	-
Culture Medium	0.5 + 0.2	-
Mouse Interferon (40 U/ml) ^b	-	49 + 1
Mouse Interferon (40 U/ml) + Anti-Mouse Interferon Serum (1:400)	-	6 + 1
Culture Medium	-	9 + 2

^aFor details, see Materials & Methods Section

^bValues were not significantly different in the presence of normal rabbit serum

Table 17 Neutralization of human leukocyte interferon by anti- γ -endorphin antiserum*

Serum (1:100 dilution)	Interferon titer, \log_{10} (units)	Reduction of interferon titer, %
Anti- γ -endorphin†	3.0	90‡
Normal rabbit	4.0	0
None	4.0	—

* Procedures are as described in Fig. 1, except a different leukocyte interferon preparation was used.

† Rabbit anti- γ -endorphin antiserum had a titer by radioimmunoassay of 1:35,000 against γ -endorphin.

‡ Difference is significant ($P < 0.01$).

Table 18. Neutralization of ACTH bioactivity by rabbit anti-human leukocyte interferon antiserum

ACTH. units/ml	Bioactivity				Normal rabbit serum 10 ⁻²	No serum
	Anti-human leukocyte interferon antiserum					
	10 ⁻²	10 ⁻³	10 ⁻⁴			
0.1	2+	3+	4+	4+	4+	
0.01	1+	2+	4+	3+	3+	
0.001	0	0	1+	2+	2+	

A 1:10,000 dilution of the rabbit anti-human leukocyte interferon antiserum neutralizes 10 units of leukocyte interferon. ACTH (Sigma) and the antisera were diluted in medium and mixed at the indicated final concentrations. The samples were incubated at 37°C for 2 hr, then assayed for ACTH activity by a mouse adrenal tumor (Y-1) cell-rounding assay. The degree of ACTH activity or cell rounding was rated on a scale of 0-4+: 0 represents the background number of rounded cells in control wells treated only with medium—approximately 32 rounded cells per $\times 100$ microscope field; 4+ represents the maximum degree of activity—approximately 300 rounded cells per field; and 3+, 2+, and 1+ represent lower numbers of rounded cells— $\frac{3}{4}$, $\frac{1}{2}$, and $\frac{1}{4}$, respectively, of the maximum.

Table 19 ACTH blockage of neutralization of human leukocyte interferon by anti-human leukocyte interferon antiserum*

Serum†		ACTH (2 units)	Inhibition of leukocyte interferon activity,‡ %
Anti-leukocyte interferon antiserum (1:200)	Normal rabbit antiserum (1:200)		
+	—	+	36
+	—	—	88
—	+	+	0
—	+	—	0

* Except for a different antiserum, procedures are as described in Fig. 1 *Right*.

† A 1:10,000 dilution of the rabbit anti-human leukocyte interferon neutralizes 10 units of leukocyte interferon.

‡ The difference between anti-leukocyte interferon antiserum with and without ACTH is significant ($P < 0.05$). Differences between anti-leukocyte interferon antiserum (with or without ACTH) and normal rabbit serum are significant ($P < 0.01$).

Table 20. Pepsin cleavage of an ACTH-like fragment from human leukocyte interferon

Sample	Treatment		Activity	
	Pepsin	Filtration	Rounded cells, no./field*	Interferon units/ml
HuLeIF	-	-	33.5 ± 2.1	1000
	-	+	49.0 ± 2.8	0
	+	-	101.5 ± 9.2	0
	+	†	73.0 ± 4.2	0
ACTH	-	-	290.0 ± 14.1	0
	-	+	71.0 ± 2.8	0
	+	-	200.0 ± 35.3	0
	+	†	74.5 ± 2.1	0
HuFbIF	-	-	40.0 ± 4.2	1000
	+	-	35.0 ± 2.1	0
Medium	-	-	32.5 ± 6.6	0
	+	-	30.0 ± 3.5	0

Human leukocyte interferon (HuLeIF), ACTH (0.01 units/ml), or human fibroblast interferon (HuFbIF) were diluted as indicated in acetate buffer (0.07 M sodium acetate/0.05M NaCl, pH 4.0), and pepsin (Sigma) was added to a final concentration of 3% (wt/wt) of the total protein (16). The mixtures were incubated for 18 hr at 37°C and then assayed on mouse adrenal tumor (Y-1) cells for ACTH activity or on human amnion (WISH) cells for antiviral activity. Some samples as indicated were passed through an ultrafilter membrane (Diaflo membrane-DM5, Amicon, Lexington, MA) with a molecular weight limit of 5000 daltons, and the filtrates were assayed for ACTH activity.

* Mean ± SD.

† The ACTH-like activity in this filtrate could be neutralized by incubating a sample with an equal volume of a 1:50 dilution of the anti-ACTH₍₁₋₁₃₎ antisera at 37°C for 2 hr prior to assay on Y-1 cells.

‡ The ACTH activity in this sample would not pass through an ultrafilter membrane (Diaflo membrane-UM2, Amicon) with a molecular weight limit of 1000.

Table 21
ACTH-like Activity from Human Leukocyte Interferon

Sample	Treatment		Rounded Cells per Field (± SD)
	Pepsin	Antisera	
Interferon (1000 U/ml)	-	-	5.0 ± 1.0
	+	-	20.5 ± 4.5
	+	anti-ACTH ₍₁₋₁₃₎	6.0 ± 1.4
	+	Normal	22.0 ± 7.1
Mock Interferon	-	-	8.0 ± 1.0
	+	-	4.5 ± 0.7
ACTH (0.001 U/ml)	-	-	85.0 ± 11.9
	+	-	72.5 ± 13.4
	+	anti-ACTH ₍₁₋₁₃₎	24.0 ± 2.8
	+	Normal	67.0 ± 0

Interferon was induced by MDV infection of human peripheral lymphocytes as previously described (18). Mock interferon is the supernatant fluid from noninfected lymphocyte cultures. Human leukocyte interferon and ACTH (Sigma Chemicals Co., St. Louis, MO) were diluted in acetate buffer (0.07M sodium acetate, 0.05 M NaCl, at pH 4.0) and pepsin (E.C. No. 3.4.4.1, No. P-7-12 Sigma Chemical Co., St. Louis, MO) was added as indicated to a final concentration of 3% (w/v) of the total protein (51). The mixtures were incubated for 18 h at 37°C. Next aliquots of the pepsin treated samples were incubated with anti-ACTH₍₁₋₁₃₎ (1/100 final dilution) or NKS (1/100 final dilution) in F-10 medium containing 10% horse serum for 1 h. All samples were then diluted at least 1:2 in F-10 medium and assayed on mouse adrenal tumor (Y-1) cells for ACTH activity (22). Rounded cells were counted per microscope field (magnification=100x).

Substance	Final Concentration (Dilution) or (M)	Inhibition of specific binding (% \pm SD)
Leukocyte interferon (1000 Units)	1:1*	47.3 \pm 2.5
	1:10	17.0 \pm 8.0
	1:100	2.3 \pm 4.6
Musk Interferon	1:1*	5.8 \pm 4.3
	1:10	5.3 \pm 4.9
	1:100	0.9 \pm 1.3
γ -Endorphin	10 ⁻⁶	51.4 \pm 2.0
	10 ⁻⁷	67.2 \pm 3.4
	10 ⁻⁸	18.0 \pm 0.6
	10 ⁻⁹	2.8 \pm 2.8
Naloxone	10 ⁻⁵	23.0 \pm 8.7
	10 ⁻⁶	76.0 \pm 12.4
	10 ⁻⁷	56.0 \pm 15.7
	10 ⁻⁸	15.8 \pm 10.6
	10 ⁻⁹	0.1 \pm 0.2

Opioid receptor binding assays were performed on the homogenized, extensively washed, particulate fraction of whole mouse brains (in 50 mM Tris-HCl buffer, pH 7.4) according to the method of Simon et al. (23). The particulate fraction was suspended in 2 ml of buffer (2% w/v) and incubated for 5 min with 0.1 ml of the indicated sample at 37°C. Next, 0.1 ml of 1,7,8-³H-dihydromorphine (New England Nuclear; 37.7 Ci mmol⁻¹, 5 \times 10⁻⁶ M) was added, the samples mixed, and incubated in the dark at 37°C for 15 min. The particulate brain material with the bound ³H-dihydromorphine was collected on a glass fiber filter (type A/E Gelman Instrument Co.; Ann Arbor, MI) and washed 3 times with 4 ml of cold buffer. The filters were dried and counted in 10 ml of liquid scintillation cocktail (Scintil Versa; Fisher Scientific Co., Fair Lawn, N.J.) in a Packard Tricarb scintillation counter. Incubations were carried out in duplicate and the values listed above represent the mean \pm the standard deviation of 3-8 experiments. Specific binding was defined as that fraction of the bound radioactivity displaced by naloxone (10⁻⁶ M). In each assay 2,000 cpm of ³H-dihydromorphine bound specifically and 1,000 cpm bound nonspecifically.

* dilution factor

Table 23

Specificity of Antisera Used for Immunofluorescent Staining

Sera	Fluorescence lymphocytes (% \pm SD)	
	Uninfected	HDV-Infected
anti-ACTH ₄ (1-13)	3.6 \pm 2.0	95.0 \pm 4.8
anti- γ -endorphin	4.4 \pm 2.3	95.7 \pm 5.1
anti-ACTH ₄ (1-13) absorbed with ACTH (10U)	3.0 \pm 1	4.6 \pm 1.4
anti-ACTH ₄ (1-13) absorbed with HPL (1 \times 10 ⁶)	5.1 \pm 2.7	98.9 \pm 6.7
anti-ACTH ₄ (1-13) absorbed with HDV	6.5 \pm 4.9	96.5 \pm 4.9
anti-HDV	4.0 \pm 1.4	100 \pm 0
anti-human immunoglobulin	18.3 \pm 5.4	19.8 \pm 2.1
Normal rabbit	5.0 \pm 2.8	1.5 \pm 0.7

Human peripheral lymphocytes (HPL) at 1 \times 10⁶ cells/ml were infected with HDV (10 HA units). At 18 h post infection, the lymphocytes were fixed on coverslips, incubated with the indicated rabbit antiserum, and then stained with FITC conjugated goat anti-rabbit IgG serum as described under Fig. 6. Three hundred to five hundred lymphocytes were observed for fluorescence.

Table 24. Neutralization of Mouse Embryo Cell Control Material by Antifibronectin Antiserum

SAMPLE	TREATMENT ^a	VIRUS TITER (pfu/0.1 ml)	% INHIBITION VIRUS CONTROL
CM ^b	—	10×10^4	71
CM	Antifib 1:10	34×10^4	1
	1:100	13.5×10^4	60
CM	NRS 1:10	7.0×10^4	80
	1:100	8.0×10^4	76
Virus Control	—	35×10^4	—

a) 0.45 ml of undilute CM was combined with 50% growth media, antifibronectin antiserum, or normal rabbit sera at the indicated dilutions. This was incubated at 37°C for 30 minutes and assayed for CM activity as before.

b) CM from human lung cells was also neutralized. Mouse L 929 and human WISH cell CM were not neutralized by antifibronectin.

Table 25. Effect of Interferon on Fibronectin-Associated Indirect Immunofluorescence in Human WISH Cells^a

<u>TIME(h)</u>	<u>INTERFERON</u>	<u>u/ml</u>	<u>FLOURESCENCE</u>
2	-	-	-
2	+	5	+
	+	15	++
	+	50	++
4	+	1	+
	+	5	++
	+	15	++
	+	50	++
6	+	1	++
	+	5	++
	+	15	++
	+	50	++
24	+	1	++
	+	5	++
	+	15	++
	+	50	++

a) Human WISH cells were grown on circular coverslips to confluency and treated with media or the indicated concentrations of human fibroblast interferon for the indicated amounts of time. Coverslips were then washed 3x in phosphate buffered saline, dried, and fixed in cold 95% ethanol for 5 min. Coverslips were then rehydrated and treated with one drop of a 1:30 dilution of rabbit antifibronectin antiserum for 30 mins. Excess antiserum was washed off and coverslips treated with one drop of a 1:15 dilution of fluorescein conjugated goat anti-rabbit antiserum (Cappel Laboratories, Cochranville, PA.) for 30 mins. The excess was washed off, slides were mounted and examined for immunofluorescence. Controls consisted of coverslips treated with normal rabbit serum plus conjugate or conjugate alone. Both controls were negative.

b) - indicates background level immunofluorescence in control cells.
+ indicates relative levels of increasing immunofluorescence.

Table 26. Effect of Fibronectin Depleted Human Fibroblast Interferon on Fibronectin-Associated Immunofluorescence in WISH Cells.^a

<u>TIME</u>	<u>INTERFERON</u>	<u>FIBRONECTIN DEPLETED</u>	<u>FLUORESCENCE^b</u>
1	-	-	-
	+	-	+
	+	+	+
3	+	-	+
	+	+	+
6	+	-	+
	+	+	+
24	+	-	+
	+	+	+
	House	-	-

- a) Coverslips prepared as before. 10 ml of Human fibroblast interferon (50u/ml) was subjected to gelatin/sepharose affinity column chromatography. This interferon was then used along with non-chromatographed interferon as before.
- b) - refers to 0-20% fluorescence
+ refers to 70-100% fluorescence

Table 27. Effect of Interferon on ³H Fibronectin in Human WISH Cells^a

<u>INTERFERON</u>	<u>³H AA cpm</u>	<u>% INCREASE</u>
None	135 ± 11	-
Mouse	71 ± 2	0
Human	345 ± 6	155

- a) Human WISH cells were grown to confluency in 250 cm² Corning tissue culture flasks. 10 ml of human fibroblast interferon (50 u/ml), mouse fibroblast interferon (50 u/ml), or growth media was added with approximately 7.5 μCi ³H amino acids. Cultures were allowed to incubate overnight at 37°C. Following incubation, cells were removed from the culture flasks with rubber policemen, centrifuged at 500 xg for 5 min and resuspended in 5 ml of growth medium. Cells were then sonicated at 40 Hz for 1 min then centrifuged at 2000xg for 5 min. The supernatant was saved and subjected to gelatin/sepharose affinity chromatography. 4M urea was used to elute the column. Collected fractions were then checked for fibronectin specific incorporated radioactivity.

Table 28. Kinetics of the Antiviral Action of CM.

Hours of treatment	% Inhibition of VSV Yield
0	0
1	0
2	0
4	0
6	80
24	80

CM was prepared by sonication of L cells (Progress Report 2). Cellular debris was removed by centrifugation and supernatant fluids (CM) were placed on human WISH cells for the indicated times. Cultures were then washed and challenged with VSV. Virus yields were determined 24 hours later.

Table 29. Inhibition of the Antiviral Action of CM by Cycloheximide

Addition	% Inhibition of VSV Yield
CM	80
CM + cycloheximide	0
cycloheximide	0

CM was prepared as described in Table 28. Human WISH cells were treated with either CM, cycloheximide (5 μ g/ml) plus CM or cycloheximide for 8 hrs. Cultures were then washed to reverse the cycloheximide block and were infected with VSV virus yields were determined 24 hours later.

Fig. 1. Induction of cell rounding and antiviral activity in mouse adrenal tumor (Y-1) cells by ACTH and interferon.

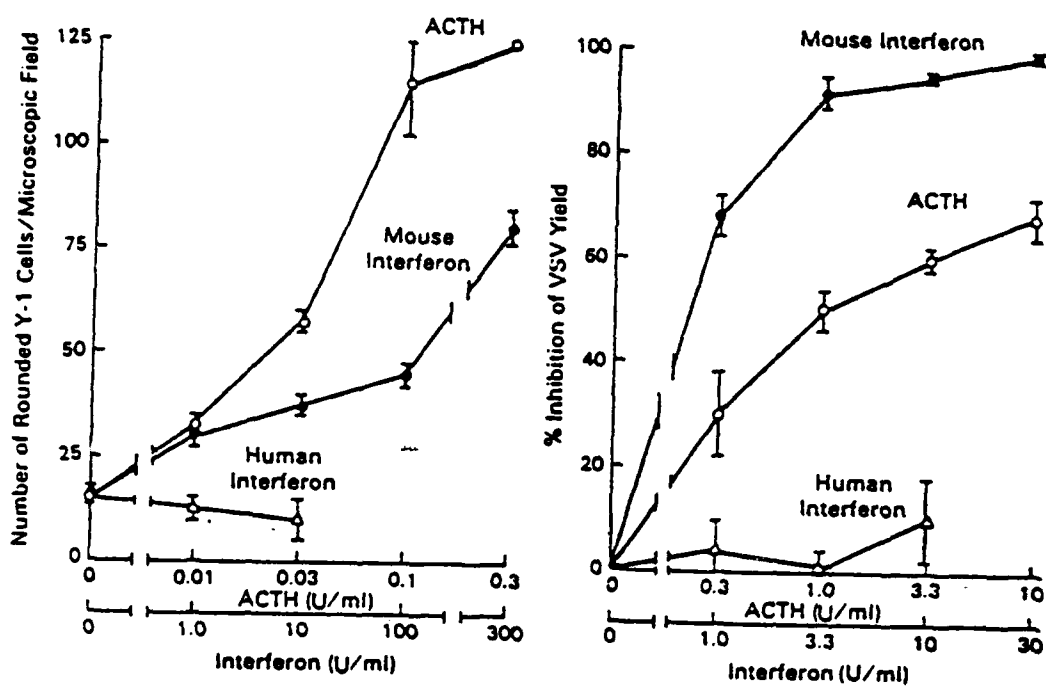
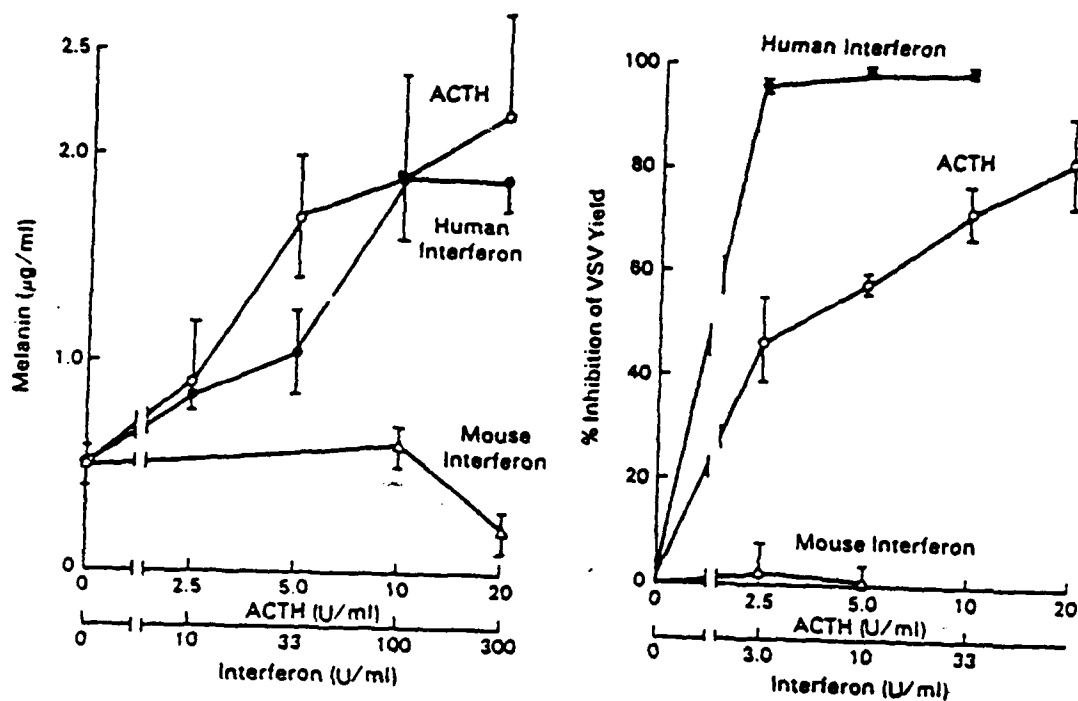


Fig. 2. Induction of melanin and antiviral activity in human melanoma (Foss) cells by ACTH and interferon.



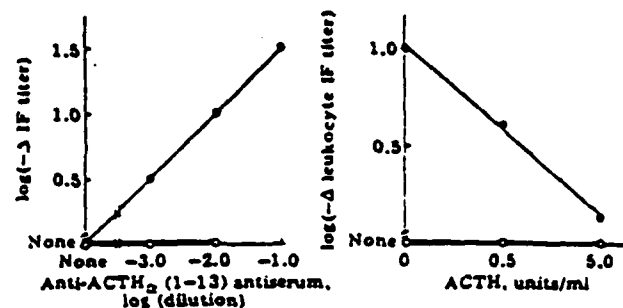


FIG. 3 Neutralization of human leukocyte interferon (IF) by anti-ACTh_n(1-13) antiserum (Left) and prevention of neutralization by ACTH (Right). (Left) Dilutions of human leukocyte (●) or fibroblast (○) interferons were incubated overnight at 4°C with the indicated concentrations of rabbit anti-ACTh_n(1-13) antiserum or normal rabbit serum. Interferon dilutions were then placed on human amnion (WISH) cells and interferon activity was determined. Both interferons in the presence or absence of normal rabbit serum had 50% plaque reduction titers of 1000. (Right) For blockage of neutralization, the indicated concentrations of highly purified ACTH were incubated with 1:50 dilutions of anti-ACTh_n(1-13) antiserum (●) or normal rabbit serum (○) for 30 min at 4°C. Equal volumes (0.15 ml) of the above were added to dilutions of human leukocyte interferon and incubated for 1 hr at 4°C. Interferon activity was determined as before. Human leukocyte interferon in the presence or absence of normal rabbit serum with or without ACTH had a 50% plaque reduction titer of 1000. All differences of 0.5 log₁₀ are significant ($P < 0.05$).

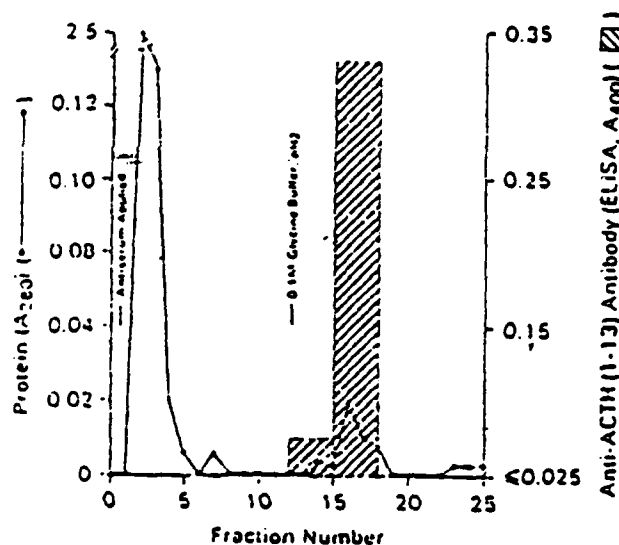


Figure 4 Purification of anti-ACTh_n(1-13) antibody by affinity chromatography on an ACIII-sepharose column.

One ml of rabbit anti-ACTh_n(1-13) antiserum was passed through a 4 ml affinity column containing sepharose 4B-bound ACTH (10 µg) (52). The column was eluted with 4 ml 0.1 M glycine buffer, pH 2. Fractions were 5 ml, except for numbers 12-25 which were 1 ml. The column fractions were pooled and assayed for anti-ACTh activity by an indirect ELISA technique (46). For the ELISA assay, 50 µg of ACTH (diluted in carbonate-bicarbonate coating buffer, pH 9.6) was adsorbed per well (overnight) and 0.2 ml of column fractions (diluted 1/5 in PBS-tween buffer, pH 7.4) was incubated for 2 h at room temperature. Next, 0.2 ml of alkaline phosphatase-conjugated goat anti-rabbit IgG (Miles Labs, Elkhart, Ind.) (1/300 in PBS-tween buffer) was incubated in the wells for 2 h at room temperature. Following 3 washes of 0.2 ml p-nitrophenyl phosphate (1 mg/ml in carbonate buffer) was incubated at room temperature for 30 min. The reaction was stopped by the addition of 50 µl of 3 M NaOH per well and the absorbance (400 nm) measured.

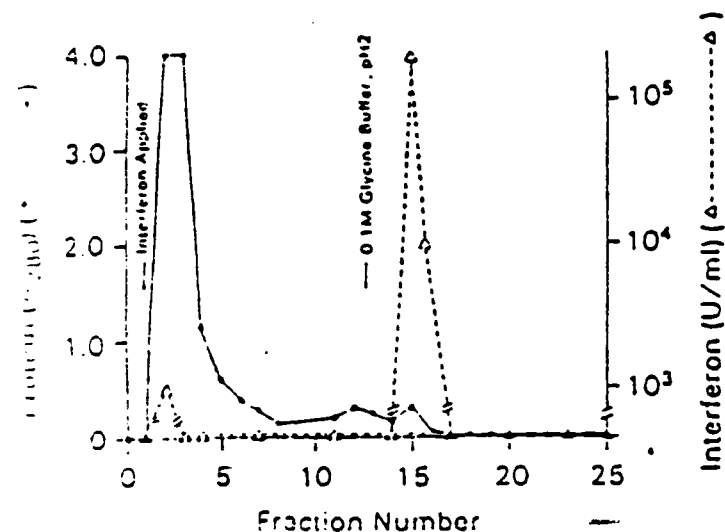


Figure 5 Purification of human leukocyte interferon by affinity chromatography on an anti-ACIH (1-13) sepharose column (52).

Interferon (U): loaded, 4.5×10^5 ; voided, 4.5×10^4 ; and eluted 10^5 (90%). Interferon was eluted with 5 ml of 0.1 M glycine buffer pH 2.0. Column bed volume was 4 ml and 5 ml fractions were collected, for number 2 which was 45 ml and numbers 9-19 which were 2 ml.

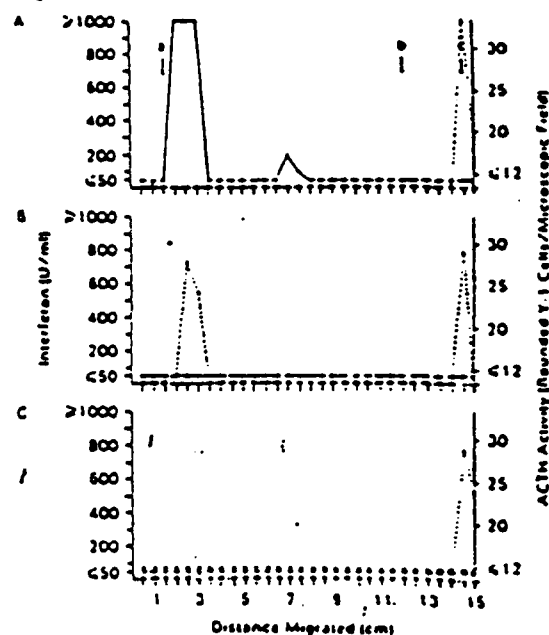


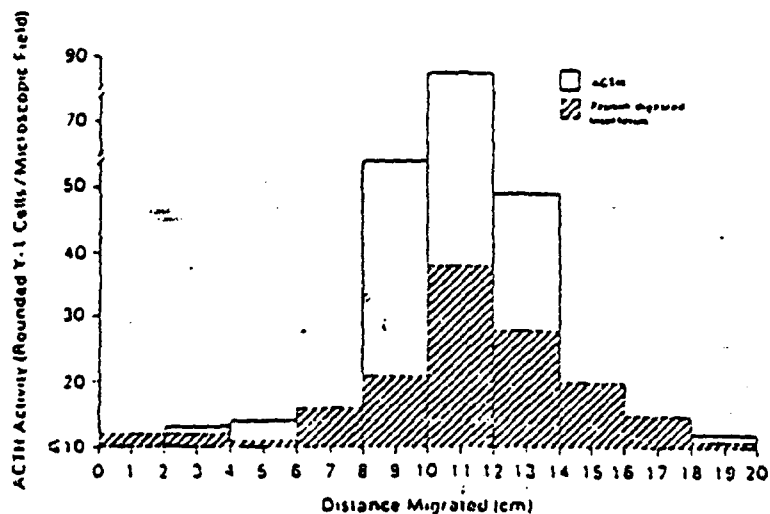
Figure 6 Polyacrylamide gel electrophoretic analysis of the association between leukocyte interferon and ACIH.

Samples for electrophoresis were prepared in 0.01 M phosphate buffered saline (PBS) at pH 6.7, containing 1.0% SDS, plus 0.4 M urea and were incubated at 25°C for 1 h. Three tenths ml samples were applied to a 12.5% cylindrical polyacrylamide gel (150 x 7cm) and electrophoresis was in a discontinuous buffer system (19) for 4 h in a constant power mode with an initial current of 5 mA per gel tube. Gels were sliced and the segments crushed and eluted into 1 ml of PBS (pH 7.2) overnight. Eluted material was assayed on mouse adrenal tumor (T-1) cells for ACIH activity (22), expressed as rounded cells per microscope field (magnification=100x). Gel samples were assayed for antiviral activity on human amnion (VISH) cells by a microplaque reduction technique (21), (Δ --- Δ), ACIH activity; (o---o), interferon activity.

- Partially purified, (as described in text) human leukocyte interferon (4×10^5 U, 10^5 U/mg protein).
- Fractions from the gel in (a) were digested with pepsin (3% of total protein) overnight and then assayed for ACIH and interferon activities.
- Large molecular weight interferon eluted from the gel in (a) was reelectrophoresed and assayed for ACIH activity (o---o) or was pepsin digested (3% pepsin, overnight), then electrophoresed and the gel slices assayed for ACIH (Δ --- Δ) or antiviral (o---o) activity. Known molecular weight standards were: a-trypsin, 24,000 daltons; b-lysozyme, 14,000 daltons; and c-ACIH, 3900 daltons.

Figure 7 Comparison of ACIH activity from human leukocyte interferon with purified ACIH by polyacrylamide gel electrophoresis.

Human leukocyte interferon was purified, digested with pepsin (3% w/w), and electrophoresed as described in Figure 4. Pepsin digested interferon (2x10⁶ U) and ACIH (6.9 U) were electrophoresed in 15% cylindrical polyacrylamide gels (200 x 7 mm) for 6 h in a constant power mode with an initial current of 5 mA per gel. The gels were sliced, eluted in PBS, pH 7.2, and assayed for ACIH activity on Y-1 cells, as described under Figure 4.



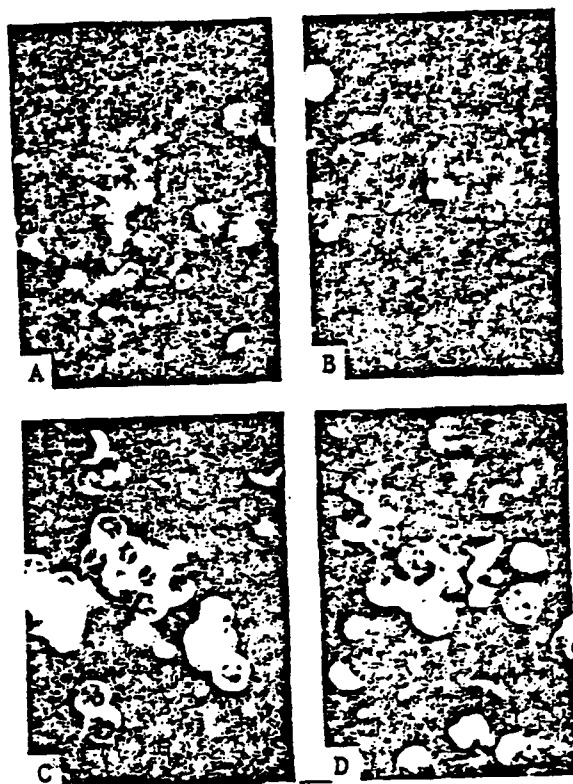


Figure 8 Immunofluorescent detection of ACTH and endorphin-like substances in MDV-infected lymphocytes.

Human peripheral lymphocytes (HPL) were separated from whole blood by centrifugation on a ficoll-hypaque gradient (17). One ml of HPLs (1×10^6 cells/ml) were inoculated with 10 hemagglutination units (HA) of Newcastle's Disease Virus (MDV, Strain 8-1). The cultures were incubated for 18 h at 37°C in Eagle's MEM supplemented with 25 fetal bovine serum. Twenty-five μ l of lymphocyte suspension (minus plastic adherent cells) were placed on a coverslip and allowed to dry. The coverslips were fixed in -4°C ethanol for 5 min. Next the coverslips were stained using a slightly modified indirect immunofluorescence procedure by Porter et al. (53). The fixed lymphocytes were rehydrated in 0.01M phosphate buffered saline (PBS) and then incubated with anti-ACTH α (1-13), anti- γ -endorphin, or normal rabbit sera (NRS) each diluted 1:50 with PBS. The anti-ACTH α (1-13) and anti- γ -endorphin sera (Bio Rix, Brussels, Belgium) were prepared in rabbits against synthetic antigens and were highly specific. Anti-ACTH α (1-13) sera has a radioimmunoassay titer of 1:38,000 against ACTH α (1-13) and a titer of 1:1000 against natural porcine ACTH (1-39). Anti- γ -endorphin has a radioimmunoassay titer of 1:35,000 against γ -endorphin. Rabbit immunoglobulins bound to the lymphocytes were detected with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Cappel Labs., Cochranville, PA.) diluted 1:15 with PBS. Coverslips were washed in PBS and mounted in a glycerol (90%) and PBS (10%) solution. The coverslips were observed through an incident light fluorescence photomicroscope (Carl Zeiss; Oberkochen, West Germany) and photomicrographs taken on Ektachrome 400 color slide film (Eastman Kodak Co.; Rochester, NY). a) MDV-infected lymphocytes at 18 h postinfection stained with normal rabbit sera (NRS), b) non-infected lymphocytes stained with anti-ACTH (1-13) sera (the fluorescing material in the upper left is nonspecific), c) MDV-infected lymphocytes at 18 h postinfection stained with anti-ACTH α (1-13) sera, and d) MDV-infected lymphocytes at 12 h postinfection stained with anti- γ -endorphin sera. Magnification=640x.

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PUBLICATIONS

The following papers have resulted from this grant:

1st Year

1. Blalock, J.E. and S. Baron. 1979. Mechanisms of Interferon-Induced Transfer of Viral Resistance Between Animal Cells. J. Gen. Virol., 42:363-372.
2. Blalock, J.E. 1979. Cellular Interactions Determine Rate and Degree of Interferon Action. Infect. Immun., 23:496-501.
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2nd Year

5. Blalock, J.E. 1979. A Small Fraction of Cells Communicate the Maximal Interferon Sensitivity to a Population. Proc. Soc. Exp. Biol. Med., 162:80-84.
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3rd Year

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